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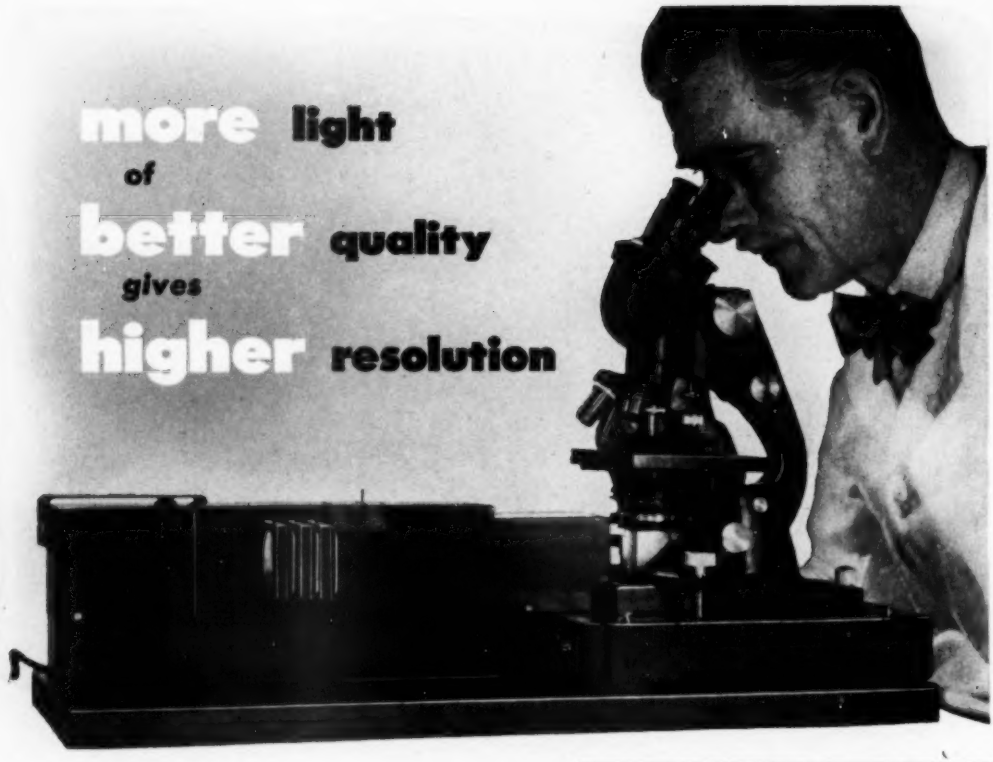

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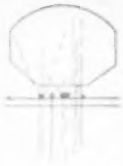

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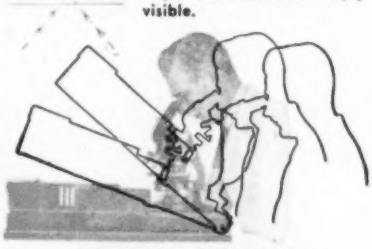

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ARCHIVES OF PATHOLOGY

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LESIONS OF THE PANCREAS IN MALIGNANT HYPERTENSION

Review of One Hundred Cases at Necropsy

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AND
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IN A PREVIOUS study [1] of dilatation of pancreatic acini in patients who died because of uremia, one of us (Baggenstoss) was impressed by the large incidence of other parenchymal lesions in those cases in which uremia was the result of nephrosclerosis. Inasmuch as the parenchymal lesions in these cases of nephrosclerosis were usually associated with severe degrees of arteriolosclerosis and arteriosclerosis, it was surmised that most of the parenchymal lesions, aside from dilated acini, were the result of the vascular changes and could be interpreted as infarcts. This study endeavors to elucidate the relationship between the vascular changes and the various parenchymal lesions.

HISTORICAL ASPECTS

A review of malignant hypertension and arteriolosclerosis provided only scattered reports on parenchymal lesions of the pancreas. To our knowledge, no series including a large number of cases of malignant hypertension has been reviewed with respect to pancreatic parenchymal lesions. A survey of several textbooks of pathology [2] revealed only brief references to vascular changes in the pancreas in malignant hypertension. Only one of these references [2a] mentioned parenchymal lesions; it stated that areas of pancreatic necrosis are not an infrequent complication of arteriosclerosis and arteriolosclerosis.

Numerous studies [3] revealed that widespread arteriolosclerosis occurs in malignant hypertension. The organs that most frequently are the seat of arteriolar

From the University of Minnesota (Dr. Hranilovich, Fellow in Pathology, Mayo Foundation), and the Section of Pathologic Anatomy (Dr. Baggenstoss), Mayo Clinic.

1. Baggenstoss, A. H.: Pancreas in Uremia: Histopathologic Study, Am. J. Path. **24**:1003 (Sept.) 1948.

2. (*a*) Anderson, W. A. D., Editor: Pathology, St. Louis, C. V. Mosby Company, 1948, pp. 624 and 909. (*b*) Bell, E. T., Editor: Text-Book of Pathology, Ed. 6, Philadelphia, Lea & Febiger, 1947. (*c*) Boyd, W.: A Text-Book of Pathology: An Introduction to Medicine, Ed. 4, Philadelphia, Lea & Febiger, 1943, p. 593. (*d*) Karsner, H. T.: Human Pathology, Ed. 7, Philadelphia, J. B. Lippincott Company, 1942.

3. (*a*) Anderson.[2a] (*b*) Boyd.[2c] (*c*) Andrus, F. C.: The Relation of Age and Hypertension to the Structure of the Small Arteries and Arterioles in Skeletal Muscle, Am. J. Path. **12**:635 (Sept.) 1936. (*d*) Engel, T., cited by Gruber, G. B.: Pathologie der Bauchspeicheldrüse, in Handbuch der speziellen pathologischen Anatomie und Histologie, edited by F. Henke, and O. Lubarsch, Springer-Verlag, 1929, Vol. 5, Pt. 2, p. 305. (*e*) Feitis, H.: Über multiple

(Footnote continued on next page)

changes are the kidneys,[4] spleen,[5] and pancreas.[6] Also involved are the arterioles of the liver,[7] gastrointestinal tract,[3l, m] skeletal muscles,[8] myocardium,[3i] and skin. In all of these studies, the emphasis was on the vascular lesions themselves. Only scant attention has been directed to resultant or concomitant alterations of the organs that undergo these arteriolar changes.

In the review of American and British literature, we encountered three references to pancreatic parenchymal changes in malignant hypertension. Klemperer and Otani,[6b] in a review of 16 cases, recorded 2 cases in which circumscribed anemic necrosis of the pancreas was noted. These authors described the gross and microscopic changes in one case in adequate detail. It was apparent from their description

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Nekrosen in der Milz (Fleckmilz), Beitr. path. Anat. **68**:297, 1921. (*f*) Fishberg, A. M.: Anatomic Findings in Essential Hypertension, Arch. Int. Med. **35**:650 (May) 1925. (*g*) Keith, N. M.: Classification of Hypertension and Clinical Differentiation of the Malignant Type, Am. Heart J. **2**:597 (Aug.) 1927. (*h*) Keith, N. M.; Barker, N. W., and Kernohan, J. W.: Histologic Studies of the Arterioles in Various Types of Hypertension, Tr. A. Am. Physicians **46**:66, 1931. (*i*) Keith, N. M.: Pathologic Studies of the Arterial System in Severe Hypertension, Proc. Staff Meet., Mayo Clin. **14**:209 (April 5) 1939. (*j*) Kimmelstiel, P., and Wilson, C.: Benign and Malignant Hypertension and Nephrosclerosis: A Clinical and Pathological Study, Am. J. Path. **12**:45 (Jan.) 1936. (*k*) Koepsell, J. E.; Kuzma, J. F., and Murphy, F. D.: Hypertensive Cardiovascular Disease (Acute) (Malignant Hypertension): Clinical and Pathological Study of 39 Cases, Arch. Int. Med. **85**:432 (March) 1950. (*l*) Lynch, K. M.: Pancreatitis: Analysis of Types and Causes, Ann. Int. Med. **14**:628 (Oct.) 1940. (*m*) Moritz, A. R.: Arteriolar Changes in Essential Hypertension, Science **80**:547 (Dec. 14) 1934. (*n*) Moritz, A. R., and Oldt, M. R.: Arteriolar Sclerosis in Hypertensive and Non-Hypertensive Individuals, Am. J. Path. **13**:679 (Sept.) 1937. (*o*) Morlock, C. G.: Arterioles of the Pancreas, Liver, Gastrointestinal Tract and Spleen in Hypertension, Arch. Int. Med. **63**:100 (Jan.) 1939. (*p*) Murphy, F. D., and Grill, J.: So-Called Malignant Hypertension: A Clinical and Morphologic Study, ibid. **46**:75 (July) 1930. (*q*) Pagel, W., and Woolf, A. F.: Aseptic Necrosis of Pancreas Due to Arterial Thrombosis in Malignant Hypertension, Brit. M. J. **1**:442 (March 6) 1948. (*r*) Pilcher, J. F., and Schwab, E. H.: Arteriolar Changes in Essential Hypertension: A Preliminary Report, Texas J. Med. **28**:665 (Feb.) 1933. (*s*) Rössle, R.: Beiträge zur Kenntnis der gesunden und der kranken Bauchspeicheldrüse, Beitr. path. Anat. **69**:163, 1921.

4. (*a*) Anderson.[2a] (*b*) Bell.[2b] (*c*) Bell, E. T., and Clawson, B. J.: Primary (Essential) Hypertension: A Study of 420 Cases, Arch. Path. **5**:939 (June) 1928. (*d*) Branch, A., and Linder, G. C.: The Association of Generalized Arteriolar Sclerosis with High Blood Pressure and Cardiac Hypertrophy in Chronic Nephritis, J. Clin. Invest. **3**:299 (Dec.) 1926. (*e*) Castleman, B., and Smithwick, R. H.: The Relation of Vascular Disease to the Hypertensive State, Based on a Study of Renal Biopsies from 100 Hypertensive Patients, J. A. M. A. **121**:1256 (April 17) 1943. (*f*) Fahr, T.: Über Nephrosklerose, Virchows Arch. path. Anat. **226**:119 (June 19) 1919. (*g*) MacMahon, H. E., and Pratt, J. H.: Malignant Nephrosclerosis (Malignant Hypertension), Am. J. M. Sc. **189**:221 (Feb.) 1935.

5. (*a*) Footnote 3*e*, *f*, *i*, *n*, *o*, and *r*. (*b*) Footnote 4*d* and *g*. (*c*) Gruber, G. B.: Pathologie der Bauchspeicheldrüse, in Handbuch der speziellen pathologischen Anatomie und Histologie, edited by F. Henke, and O. Lubarsch, Berlin, Springer-Verlag, 1929, Vol. 5, Pt. 2, p. 305.

6. (*a*) Gerlei, F.: Über die Veränderungen der Bauchspeicheldrüse bei Herzkranken, Virchows Arch. path. Anat. **276**:148 (Feb. 17) 1930. (*b*) Klemperer, P., and Otani, S.: "Malignant Nephrosclerosis" (Fahr), Arch. Path. **11**:60 (Jan.) 1931. (*c*) Footnote 3*f*, *i*, *k*, *n*, *o*, *q*, and *r*. (*d*) Footnote 4*d* and *g*.

7. Footnote 3*h*, *i*, *k*, *n*, *o*, and *r*. Footnote 6*b*.

8. Kernohan, J. W.; Anderson, E. W., and Keith, N. M.: The Arterioles in Cases of Hypertension, Arch. Int. Med. **44**:395 (Sept.) 1929. Scott, R. W.; Seecof, D. P., and Hill, A. A.: Arteriolar Lesions of Skeletal Muscle in Hypertension, Tr. A. Am. Physicians **48**:283, 1933. Footnote 3*g*, *k*, and *m*.

that the lesions were ischemic infarcts. Another report was that of Pagel and Woolf,[3q] in which a detailed account was given of malignant hypertension in a 45-year-old man. Necropsy revealed a swollen pancreas that displayed increased consistency. Multiple small anemic infarcts were present in the pancreas, the largest of which measured 3.2 by 1.2 cm. The infarcted tissue was sharply demarcated by a narrow zone of hemorrhage. No evidence was present of fat necrosis or diffuse hemorrhagic pancreatitis. The small arteries were severely sclerotic, and many were occluded by thrombi. As no evidence was noted to indicate the presence of embolization, the process was considered to be one of thrombosis.

Burn [9] studied the relation of acute pancreatitis to acute coronary thrombosis. In an unstated number of necropsies on persons of all ages, the incidence of acute pancreatitis was 10%. The greatest incidence was associated with cardiac disease. It was not clear, however, how many of the persons studied had malignant hypertension or in how many of those who had coronary thrombosis, or myocardial infarction, or both were thromboembolic phenomena the basic mechanism. Terms such as "acute pancreatitis," "focal pancreatitis," and "pancreatitis" were not defined in Burn's presentation.

Somewhat more attention to pancreatic parenchymal lesions accompanying or caused by arteriolosclerosis was evident in the German literature. Engel [3d] reported a case in which infarctive pancreatic necrosis was present. The large and small arteries had thick walls, narrowed lumina, subintimal hyalinization, and degeneration of the media. Gerlei [6a] considered changes in the pancreas associated with cardiac disease. The descriptions in this report, although they reveal the role of vascular disease and thromboembolic processes in pancreatic necrosis, did not refer to arteriolar lesions as such. Feitis,[3e] in a description of multiple splenic infarcts in cases of primary renal atrophy, stated that Herxheimer did not find infarcts and necrosis of the pancreas in his series of cases of hypertension.

Gruber [5c] gave perhaps the most succinct presentation of changes in the pancreatic vasculature. According to Gruber, Fahr denied the occurrence of arteriolosclerosis in the pancreas in association with secondary renal atrophy. More will be said about this later. Gruber discussed a case described by Chiari in which pancreatic necrosis associated with granular atrophy of the kidneys was found at necropsy in a 45-year-old woman. In his own studies, Gruber did not encounter calcification in the walls of pancreatic vessels. Later reference will be made to this.

Rössle [8a] gave a detailed description of the pancreas in the case of a 52-year-old man who died of peritonitis. This man had received antisyphilitic therapy. Syphilitic aortitis was evident at necropsy. It is possible that the pancreatic vascular and parenchymal changes resulted from syphilis and not from primary vascular disease. Rössle, however, was of the opinion that the infarctive process in the pancreas was entirely analogous to the usual infarcts encountered in the kidney and spleen. In his comment, he stated that Blume-Beneke and Chiari had described antemortem autodigestion of the pancreas on an ischemic basis.

In view of the lack of any thorough studies of pancreatic parenchymal lesions as a result of vascular disease, it was decided to study the pancreases in a large

9. Burn, C. G.: The Association of Acute Pancreatitis with Acute Coronary Thrombosis, Am. J. Path. **27**:680 (July-Aug.) 1951.

number of cases of malignant hypertension. It was hoped that such a study would uncover information regarding the incidence and significance of the pancreatic parenchymal lesions and their relation to vascular disease.

MATERIALS AND METHODS

The records in 100 cases of malignant hypertension encountered from 1924 through 1948 and in which necropsies were done were obtained from the files of the Section of Pathologic Anatomy at the Mayo Clinic. The clinical charts were abstracted for data referable to age; sex; onset; duration and degree of hypertension; symptoms referable to cerebrospinal, cardiovascular, and renal systems; alterations in chemical constituents of blood and other body fluids, and type of death. The necropsy protocols furnished data concerning weights of the hearts and kidneys and changes in other organs. Gross descriptions of the pancreas were studied in every case. The routine sections of the pancreas were reviewed. These were paraffin sections, 7 to 8 μ in thickness, stained with hematoxylin and eosin. In a few cases, staining technics to demonstrate collagen and elastic tissue were used.

The controls consisted of 100 cases in which the sex and the age distribution by decades of life were essentially the same as those in the cases of malignant hypertension. Clinical charts, correspondence, and necropsy protocols in the control cases were used to rule out hypertension and congenital or acquired cardiac disease. It has been stated that cardiac weight is the most valid single criterion in the evaluation of the presence or absence of hypertension. Smith,[10] in 1928, studied the relation of cardiac weight to body weight and to age. In the present study we adhere closely to his values. No case was used in which cardiac weight exceeded the maximal normal cardiac weight, as determined from the tables of Smith, by 50 gm. We also examined sections of the pancreases from 80 persons who had chronic glomerulonephritis and who died of uremia. These were examined to check on the incidence of similar pancreatic parenchymal and vascular lesions and to evaluate their real or apparent specificity.

The following lesions of the pancreas were looked for and classified as absent, slight, moderate, or severe: arteriosclerosis in large and small arteries; arteriolosclerosis; infarction; parenchymal necrosis; atrophy of parenchyma; fibrosis, both interlobular and intralobular; hemorrhage; inflammation, both acute and chronic; fat necrosis; metaplasia of ductal epithelium; and dilatation of acini, or ducts, or both.

The degree of arteriolosclerosis was evaluated on the basis of luminal narrowing. This was severe when the lumen was reduced to one-third or less of its normal diameter, moderate when it was reduced to between two-thirds and one-third, and minimal when the lumen was narrowed by less than one-third.

The criteria used for the diagnosis of malignant hypertension vary widely. According to Bell,[2b] malignant hypertension is chronic hypertension associated with uremia. This definition was favored by Karsner,[2d] Boyd,[2e] Fahr,[4f] and Anderson.[2a] Several authors implied a difference in the rate of progression of vascular lesions.[11] Keith [3g] limited use of this term to severe hypertension in conjunction with evident papilledema. This concept is generally accepted at the clinic and is the one used in this paper. Study of cases in this category usually revealed the presence of uremia and progressive vascular changes.

RESULTS

The vascular changes in the pancreas are summarized in Table 1. In the cases of malignant hypertension, arteriolosclerosis was absent in only 14% and was moderate to severe in 68%. This lesion was present in 7% of the controls, in none of which were moderate or severe degrees of arteriolosclerosis present. Moderate or severe arteriosclerosis was noted in 44 of the 47 cases of malignant hypertension

10. Smith, H. L.: Relation of Weight of Heart to Weight of Body and of Weight of Heart to Age, Am. Heart J. **4**:79 (Oct.) 1928.

11. Shapiro, P. F.: Malignant Nephrosclerosis: Pathogenesis, Arch. Int. Med. **48**:199 (Aug.) 1931. Anderson.[2a] Moritz.[3m] Gruber.[5c] Klemperer and Otani.[6b]

in which severe arteriolosclerosis was present. Minimal or no arteriolosclerosis was present in only 8 of the 56 cases of malignant hypertension in which severe arteriosclerosis was encountered. Thus it would appear that in malignant hypertension both arteries and arterioles are affected. Of the 80 cases of chronic glomerulonephritis, no arteriolosclerosis was found in 52 (65%), and moderate or severe lesions were noted in 21 (26%). A slight degree of arteriolosclerosis was encountered in the remaining seven cases (9%).

Changes, other than luminal narrowing, that were encountered in the arterioles merit further description. Many of the arterioles in which luminal narrowing was judged to be minimal showed definite homogeneous eosinophilic alterations in staining characteristics. These alterations may well represent extremely early changes in arteriolosclerosis was encountered with those in which severe arteriolosclerosis This was well demonstrated in arterioles sectioned longitudinally and in those cut in cross section. In the latter, the eccentric focal thickening resulted in an elliptic or otherwise distorted lumen (Fig. 1*a* and *b*).

In two of the cases, degeneration severer than that just described occurred in the walls of arterioles. In these the eosinophilic material was not structurally homo-

TABLE 1.—*Incidence of Pancreatic Arteriolosclerosis in Cases of Malignant Hypertension and Chronic Glomerulonephritis*

Arteriolosclerosis	Malignant Hypertension (100 Cases), No.	Chronic Glomerulonephritis (80 Cases)		Controls (100 Cases), No.
		No.	%	
Absent	14	52	65	93
Slight	18	7	9	7
Moderate	21	11	14	0
Severe	47	10	12	0

geneous but appeared to be present in a granular or globoid form (Fig 1*c*). This process appeared to be a hyaline necrosis. No evidence of necrotizing arteriolitis was found in any of the cases. We deemed necessary for the latter diagnosis the presence of necrosis or degeneration plus evidences of inflammation. As the latter component was lacking, the diagnosis was not made.

We were interested in a comparison of those cases in which minimal or no arteriolosclerosis was encountered with those in which severe arteriolosclerosis was present. In the former group were 32 cases and in the latter, 47. Clinical data regarding age, duration of disease, blood pressure (average, maximal and minimal ranges), and values for urea and creatinine in the blood were compared. No significant differences could be detected between these two groups. The mean age of patients in the former group was 44 years, whereas the mean age of those in the latter group was 49 years. Thus it would appear that a severer degree of sclerosis generally occurred in the older patients.

The small arteries exhibited changes similar to those described for the arterioles, but more often they revealed the same appearance found in arteries of larger size. These changes consisted of hypertrophy of the media and symmetric or asymmetric fibrous intimal proliferation.

Thrombosis of small or medium-sized arteries was encountered in 15 cases. In a few of these, hemorrhage was evident in the wall of the artery (Fig. 1*d*). The necropsy protocols and clinical abstracts in these 15 cases were reviewed. Of the

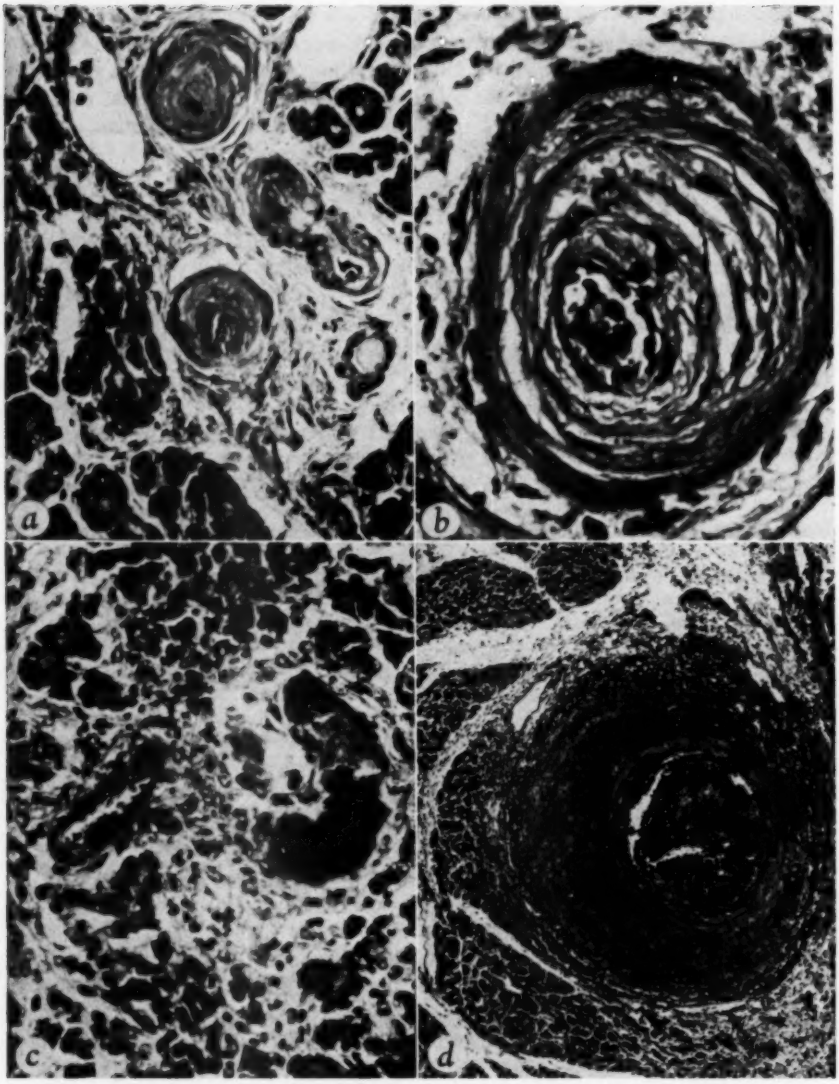

Fig. 1.—Lesions of pancreatic arterioles and arteries in malignant hypertension. (*a*) Severe hyaline sclerosis; note eccentric and distorted lumina (hematoxylin and eosin; × 280). (*b*) Intimal fibroblastic proliferation and edema (hematoxylin and eosin; × 475). (*c*) Hyaline necrosis of pancreatic arteriole. Note swelling of wall, granularity, and deep staining of hyaline substance; no intramural inflammation is present. Note surrounding zone of edema with only a rare inflammatory cell present (hematoxylin and eosin; × 300). (*d*) Artery of moderate size with thrombosis and intimal hyalinization; degeneration and hemorrhage is present through the entire wall. Note surrounding zone of hemorrhage and inflammation (hematoxylin and eosin; × 45).

15, the lesions in 2 cases possibly could be considered thromboembolic in origin; marantic thrombi were present on the mitral valves in these two cases. No apparent source for embolization was found in the 13 remaining cases.

Fine to coarse granular deposits were noted in the walls of small or medium-sized arteries in 7 of the 100 cases of malignant hypertension. This material took a deep basophilic stain when exposed to hematoxylin and eosin. We interpreted the material to be deposition of salts of calcium. This change was not evident in the controls or in the cases of chronic glomerulonephritis.

Focal infarcts of the pancreas occurred in seven cases (Table 2). We defined an infarct as a region of necrosis in tissue that resulted from obstruction of the circulation to the region. Rose [12] and others [2b, c] stated that the vascular obstruction must be acute or rapid. Others, such as Anderson,[2a] do not specify as to the rate of obstruction. The largest pancreatic infarct noted grossly was approximately 3 cm. in its greatest diameter. The infarcts were soft, red to reddish yellow, and surrounded by a zone of hemorrhage. Histologically, evidence of necrosis of all cellular elements in the region was present, along with severe extravasation of

TABLE 2.—*Incidence of Pancreatic Lesions in One Hundred Cases of Malignant Hypertension and One Hundred Controls*

Lesions	Malignant Hypertension	Controls
Infarction	7	2
Parenchymal necrosis	21	2
Necrosis of fat	10	2
Hemorrhage	23	7
Inflammation		
Acute	39	3
Chronic	8	1
Capillary-venous dilatation	22	15
Metaplasia of ductal epithelium	17	15

erythrocytes, a peripheral zone of acute inflammation, and dilatation of neighboring vessels (Fig. 2). We also noted relatively large regions of tissue at or near the margins of some of the infarcts that were somewhat different. Here, the parenchymal cells appeared to be shrunken, the nuclei were hyperchromatic and pyknotic, and apparent separation had occurred between the cells and basement membrane; general increase had taken place in the interstitial spaces without any apparent increase in substance (Fig. 3). This appearance suggested an early phase in edema and death of tissue because of ischemia. Evidence of inflammation was minimal or absent, and erythrocytic extravasation was not a prominent feature. The appearance was analogous to that seen at the peripheries of infarcts in other organs, such as the heart, in which such borderline changes in tissue may be noted. Thrombosis of small or medium-sized arteries was demonstrable in six of these seven cases. In one case, vascular thrombosis was not found in the sections searched. In another case, an old canalized thrombus was encountered, but nothing was present to suggest healed or recent pancreatic infarction.

Lesions that we designated as parenchymal necrosis were present in 21 cases of malignant hypertension (Table 2). This lesion was encountered only twice in the controls. As already indicated, we reserved the term "infarct" for those lesions

12. Rose, W. M.: Studies in Pathology, London, Cambridge University Press, 1950, p. 171.

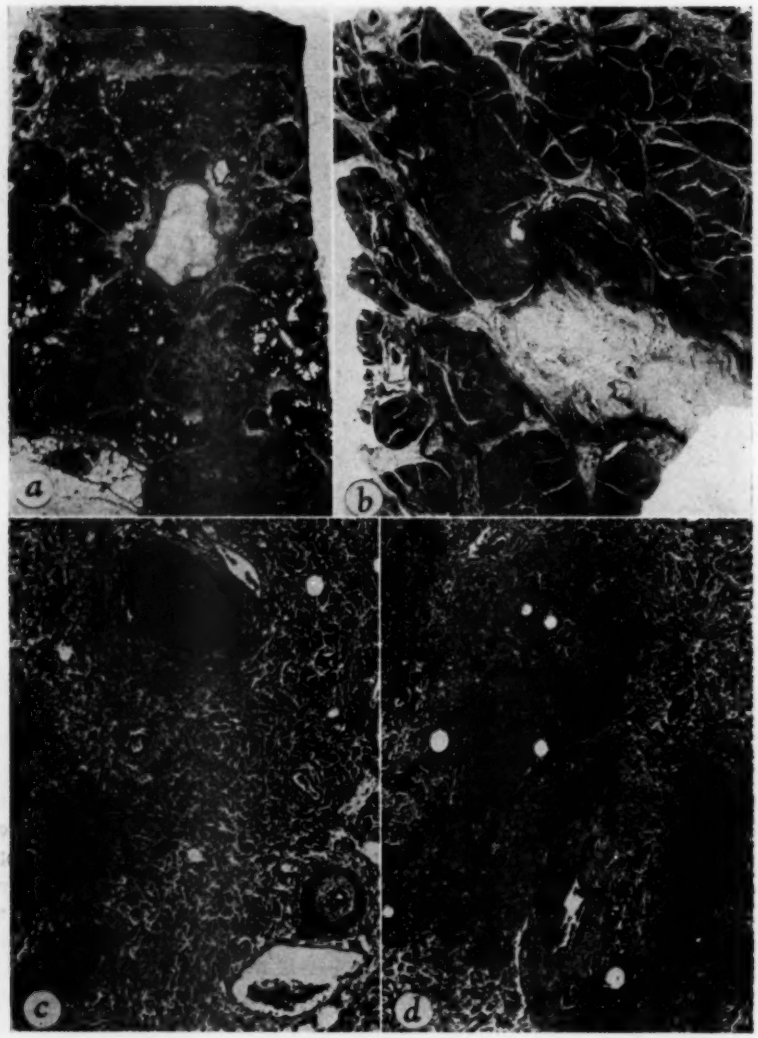

Fig. 2.—Infarcts of pancreas. (*a*) Parenchymal necrosis in center; note widespread fibrosis and foci of parenchymal atrophy. A thrombosed artery is seen in the lower left portion of the Figure. In the right lower field is a dark area that represents a recent infarct; the lighter zone around this dark area has same appearance as areas of focal necrosis seen in Figure 3 *a* (hematoxylin and eosin; × 7). (*b*) Large area of necrosis with inflammation at its periphery. In the upper and lower left portions of the field are areas of more recent infarction; note thrombosed vessel in the upper part of the midfield (hematoxylin and eosin; × 6). (*c*) Pancreatic arterial thrombosis; infarction is present in the lower left. The thrombosed artery shows hemorrhage in the wall; note intimal proliferation in the artery in the right lower field (hematoxylin and eosin; × 45). (*d*) Another region from same pancreas pictured in Figure 2*c* (hematoxylin and eosin; × 45).

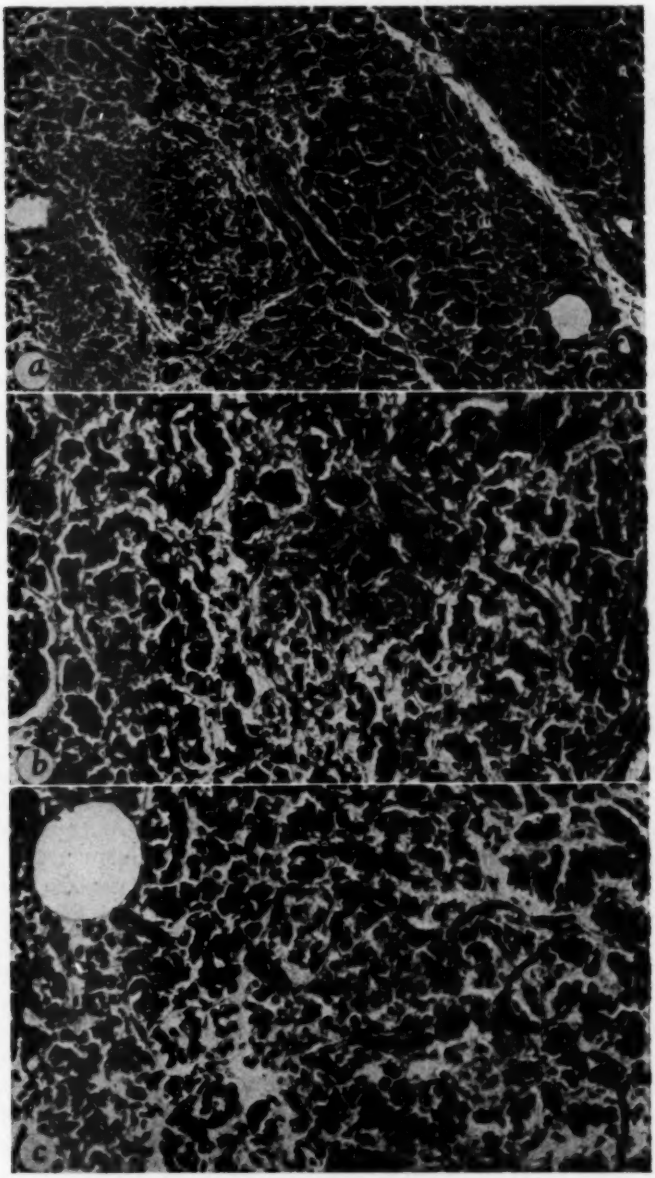

Fig. 3.—Focal parenchymal necrosis. (*a*) Altered architecture, cellular disintegration, and karyorrhexis; note absence of hemorrhage and lack of prominent inflammation (hematoxylin and eosin; × 100). (*b*) Small focus showing disappearance of nuclei, pyknosis and loss of cellular outline (hematoxylin and eosin; × 200). (*c*) Necrosis of an islet of Langerhans. Cellular outlines are indistinct, and the cytoplasm is coarsely granular; note nuclear ghosts in center, with pyknosis and hyperchromatism of the more peripherally located nuclei. The capillaries are slightly dilated and engorged (hematoxylin and eosin; × 200).

which, in addition to necrosis revealed hemorrhage and acute inflammation. As was intimated, however, changes took place in or near regions of infarction that were histologically different and that suggested ischemic death of tissue without all the signs of obvious infarction (Fig. 3*a*). In none of these 21 patients were the lesions of parenchymal necrosis large enough to be recognized grossly. The largest involved one-half to two-thirds of a lobule, but most lesions included only 6 to 10 acini. In every case the lesions were focal and numbered from one to four per section. The histologic features were variable. Some lesions displayed pyknosis and hyperchromatism of nuclei, decrease in cellular size, separation of cells from one another, interstitial edema, distortion of acinar patterns, and minimal inflammation (Fig. 3*a* and *b*). These lesions displayed parallelism to peripheral regions in other sections that showed obvious infarction of pancreatic parenchyma. On the other hand, several of the lesions revealed faintly eosinophilic, granular degeneration of acini. The nucleus had completely disappeared, but the cellular outline still was apparent. Dissolution of cells was evident in a few of the affected regions. In none was hemorrhage, congestion, or inflammation a prominent feature. In every case in

TABLE 3.—*Incidence of Pancreatic Parenchymal Atrophy in One Hundred Cases of Malignant Hypertension and One Hundred Controls* *

	Type			
	Focal		General	
Degree	Malignant Hypertension	Controls	Malignant Hypertension	Controls
Slight	31	8	8	0
Moderate	10	4	5	0
Severe	18	0	1	0

* Atrophy was absent in 27% of cases of malignant hypertension and in 88% of the controls.

which parenchymal necrosis was found, arteriosclerosis and arteriolosclerosis were judged to be of severe grade, but thrombosed vessels were not observed. It is our opinion, after careful scrutiny of these necrotic lesions, that they represent minute ischemic infarcts of the pancreatic parenchyma. In seven cases, not noted in Table 2, lesions of the islets of Langerhans were encountered. The histologic features, in essence, were similar to those described for focal necrosis of the parenchyma (Fig. 3*c*). In five of these seven cases, these lesions were associated with other parenchymal changes, whereas in two cases no other parenchymal lesions were noted, although a search for them was conducted.

Fat necrosis, as typically described in textbooks, was present in 10 cases. In four of these cases, our sections did not reveal an associated necrosis of parenchyma. Histologically, the fat necrosis was the same, whether or not parenchymal necrosis was present. Perhaps study of additional sections would have revealed concurrence of parenchymal and fat necrosis in all cases.

Foci of parenchymal atrophy were present in 73 of the 100 cases of malignant hypertension but in only 12 of the controls (Table 3). It was difficult to evaluate and grade parenchymal atrophy. The lesion was classified in two ways, namely, degree and distribution (generalized or focal). In an initial survey we established maximal and minimal degrees of involvement on the basis of amount of loss of parenchyma and fibrous replacement in the entire section. We then graded the

degrees between these extremes. In almost every case, atrophy of the parenchyma was accompanied by a relatively proportionate increase in connective tissue. Exceptions were noted in those cases in which atrophic changes were so recent that insufficient time had elapsed for fibrosis to develop. Moderate or severe generalized atrophy was encountered in six cases of malignant hypertension, whereas this lesion was not seen in the controls. Moderate or severe focal atrophy was present in 28 cases of malignant hypertension, whereas this feature was encountered only four times in the controls.

Interlobular fibrosis occurred in 38 and intralobular fibrosis in 76 of the 100 cases of malignant hypertension. In the controls, the incidence of these two types of fibrosis was 16 and 25, respectively (Table 4). Regions of parenchymal atrophy were present in 73 of the 76 cases in which intralobular fibrosis occurred. We used the same method to evaluate fibrosis that was described for parenchymal atrophy. One examiner evaluated all sections, and we assumed that any error would be consistent throughout the entire series. Intralobular fibrosis, even of severe degree, was focal in distribution in the majority of the cases. The same was true to a less extent of interlobular fibrosis.

TABLE 4.—*Incidence of Pancreatic Fibrosis in One Hundred Cases of Malignant Hypertension and One Hundred Controls*

	Type			
	Interlobular		Intralobular	
Degree	Malignant Hypertension	Controls	Malignant Hypertension	Controls
Absent	62	84	24	75
Present	38	16	76	25
Slight	17	10	38	16
Moderate	12	4	20	9
Severe	9	2	18	0

Dilatation or engorgement, or both, of islet capillaries was not generalized; only a few islets in each section exhibited this feature. In general, it was associated with dilatation of capillaries and veins elsewhere in the section and would appear to be the result of passive congestion. Hemorrhage was minimal and characterized by extravasation of erythrocytes into the interlobular connective tissue. In only a few cases was it noted within the connective tissue of the lobules. If we omit cases in which obvious infarction was present, mild hemorrhage was still three times as frequent in malignant hypertension as in the control cases.

Acute inflammation was present in all cases in which infarction or fat necrosis was noted, and in some of the cases in which parenchymal necrosis occurred. The preponderance of the last three lesions named in malignant hypertension explains the different incidence of acute inflammation in this disease and in the controls.

Metaplasia of ductal epithelium was encountered in 17 cases of malignant hypertension and in 15 controls. In only two cases in the former group was the metaplasia of such degree that obstruction could occur. The difference in incidence of this lesion in the two groups is not appreciable.

Replacement of tissue by fat was present in 38 cases of malignant hypertension and in 12 controls (Table 5). It was not in itself a prominent feature. It is well to note that what was considered as a severe degree of replacement by fat applies

only to this study; more severe degrees may occur in other diseases. Our evaluation would be equivalent to grading on a curve, but it is valid with respect to this study. Our results show a clear-cut difference of incidence in malignant hypertension and in the control cases.

The occurrence of acinar dilatation in uremia has been described by one of us (Baggenstoss [1]); criteria for the evaluation of the degree of dilatation are set forth in that paper. The findings in the present study closely parallel those in the paper referred to, which is to be expected as uremia was present in the majority of our cases. Table 6 summarizes our findings with reference to dilatation of the acini.

TABLE 5.—*Incidence of Replacement of Pancreatic Tissue by Fat in One Hundred Cases of Malignant Hypertension and One Hundred Controls*

Replacement by Fat	Malignant Hypertension	Controls
Absent	62	88
Present	38	12
Slight	21	8
Moderate	13	4
Severe	4	0

TABLE 6.—*Incidence of Dilatation of Pancreatic Acini in Cases of Malignant Hypertension and Chronic Glomerulonephritis*

Acinar Dilatation	Malignant Hypertension (100 Cases)	Chronic Glomerulonephritis (80 Cases)	Controls (100 Cases)
Absent	65	52	80
Present	35	33	20
Slight	21	10	12
Moderate	10	9	7
Severe	4	5	1

COMMENT

This study at necropsy of the pancreatic lesions in 100 cases of malignant hypertension corroborates the conclusions of others,[13] namely, that severe pancreatic arteriolosclerosis occurs frequently in this disorder. In our opinion the vascular changes are an important cause in all the parenchymal lesions noted except metaplasia of ductal epithelium and acinar dilatation.

Rössle [38] suggested that vascular lesions would be found as the explanation for necrosis of fat if they were more closely looked for. This study appears to add strength to his observation. We are of the opinion that ischemia of tissue is the basic pathophysiologic process in the production of the various lesions noted. It is generally recognized that arterial thrombosis usually produces rapid changes in tissues that result in infarction. The relationship between arterial thrombosis and pancreatic infarction is illustrated in 6 of the 100 cases of malignant hypertension. On the other hand, we were unable to demonstrate thrombosis or embolism as a factor in any of the cases in which parenchymal necrosis was noted, although vascular sclerosis was severe in every case. Rose [12] and others have pointed out that acute

13. Footnote 2 *a*, *b*, and *c*. Footnote 3 *f*, *i*, *n*, *o*, and *q*. Footnote 4 *d* and *g*. Footnote 6 *a* and *b*.

arterial occlusion is not necessary to produce infarction of tissue. It is fairly certain that other factors, in addition to vascular changes, play a role. Probably of great significance among these factors are congestive cardiac failure and shock. Congestive failure and shock are frequently accompanied by a decrease in blood pressure, especially when the basis is acute myocardial infarction. This may embarrass the local circulation sufficiently to produce local anoxia with focal necrosis or infarction of the pancreas in the absence of a completely occluded vessel.[12]

Although the importance of vascular factors, both local and general, in the production of necrosis and infarction is generally recognized, this cannot be said for focal atrophy and fibrosis, which also were common lesions in these cases. In malignant hypertension, the latter two lesions are also probably produced by local ischemia. In their production, however, the local ischemia probably develops more slowly and is related to the luminal narrowing found in the arteries and arterioles. Consequently, instead of the necrosis and hemorrhage that are features of the acute "red" infarct, foci of atrophy are present with secondary replacement by fibrous and fatty tissue. That the foci of atrophy and fibrosis are not the residual lesions of previous acute infarctions is indicated by the presence of normal-appearing islets and scattered acini in the fibrotic region. In those cases in which acute infarction was noted, all structures were necrotic. The common factor in the production of necrosis and infarction, on one hand, and atrophy with fibrosis, on the other, is ischemia. The rate of development of the ischemia, however, is apparently important in the determination of which of the lesions is to develop.

As was indicated previously, Gruber [5c] had attributed to Fahr a statement that arteriolosclerosis of pancreatic vessels did not occur in any cases but those of primary renal atrophy. In our previously mentioned evaluation of the vascular changes in the pancreases in 80 cases of chronic glomerulonephritis associated with terminal uremia, we stated that moderate or severe degrees of arteriolosclerosis were present in 21 cases (26%). Pancreatic parenchymal infarction and necrosis were encountered in these cases only when severe arteriolosclerosis was present. It would be of interest to investigate a similar problem with respect to hypertension caused by other known factors. It is our opinion that arteriolosclerosis would be found in pancreatic vessels but perhaps in a smaller number of cases. The results of Barker [14] tend to support this conclusion. Arteriolosclerosis in organs other than the kidney has, according to others, served as a strong indication of the presence of hypertension. We concur with this but point out that it is by no means pathognomonic.

The question of the relationship of vascular disease to acute hemorrhagic pancreatitis is one that is frequently raised. Several authors [15] presented "vascular factors" in their tables of etiologic factors. None, however, gave a clear demonstration to support inclusion of such factors in the tables. The reports of Lynch [31] and of Pagel and Woolf,[3q] together with the experimental work of Smyth,[15c] would

14. Barker, L. F.: Arterial Hypertension Associated with Marked Hypertrophy of the Muscular Coats of the Arterioles Rather Than with Arteriosclerosis in a Patient with Secondarily Contracted Kidneys, M. Clin. North America **14**:219 (July) 1930.

15. (*a*) Lewison, E. F.: Acute Pancreatitis: An Etiologic Review and Report of 35 Cases, Arch. Surg. **41**:1008 (Oct.) 1940. (*b*) McWhorter, G. L.: Acute Pancreatitis: Report of 64 Cases, ibid. **25**:958 (Nov.) 1932. (*c*) Smyth, C. J.: Etiology of Acute Hemorrhagic Pancreatitis with Special Reference to Vascular Factors: Analysis of Autopsies and Experimental Investigation, Arch. Path. **30**:651 (Sept.) 1940.

indicate that vascular factors as such are not important in acute hemorrhagic pancreatitis. All the lesions of the pancreas alluded to in the literature as being caused by vascular changes were focal in nature. In none of the 100 cases reviewed for this study was diffuse hemorrhagic pancreatitis or diffuse pancreatic necrosis encountered. All the lesions were focal, and the majority were of microscopic nature. We would conclude, therefore, that vascular lesions alone are not important etiologic factors in the production of hemorrhagic pancreatitis.

We are of the opinion that the pancreatic lesions described in this study are an expression of severe vascular disease and that similar lesions may well occur in other organs. Such lesions, though focal and microscopic, may well partially account for the rapid and at times sudden failure of multiple organs and systems in patients who have malignant hypertension.

SUMMARY AND CONCLUSIONS

Routine histologic sections of the pancreas obtained at necropsy in 100 cases of malignant hypertension have been reviewed and correlated with analyses of clinical data in the cases.

Moderate to severe arteriolosclerosis occurred in 68 of the cases. Infarcts of the pancreas were encountered in 7 cases, focal parenchymal necrosis in 21 cases, and foci of atrophy in 73 of these 100 cases of malignant hypertension.

Vascular alterations were the most important factor in the production of parenchymal lesions such as infarction, focal parenchymal necrosis, atrophy, and fibrosis. Although arterial thrombosis could be demonstrated in 6 of the 7 cases of pancreatic infarction, it could not be demonstrated in any of the 21 cases in which parenchymal necrosis was noted. It is suggested that congestive cardiac failure and shock in the presence of severe arteriolosclerosis may be important factors in the production of parenchymal necrosis.

Arteriolosclerosis of the pancreas is strongly indicative of, but not pathognomonic for, malignant hypertension.

Vascular factors by themselves are not important etiologic factors in acute hemorrhagic pancreatitis.

PATHOLOGY OF MUMPS VIRUS MENINGOENCEPHALITIS IN MICE AND HAMSTERS

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ALTHOUGH meningoencephalitis is a common manifestation of mumps infection, it is not a well-defined pathologic entity. In man, mumps involvement of the central nervous system may be accompanied by marked clinical signs, but fatalities are rare, and, in those which have been reported, the evidence that mumps virus was the cause of death has been inconclusive.[1] The intracerebral inoculation of mumps virus into rabbits,[2] monkeys,[3] cats,[4] and other animals [5] has not been reported to cause striking illness. Gordon [6] reported a fatal mumps encephalitis in monkeys, but subsequent studies have failed to reproduce this result, and it was not clearly established that the deaths in question were due to mumps. Repeated attempts in this laboratory to induce a clinically recognizable mumps encephalitis in monkeys have not been successful.

The histologic changes in the central nervous system most frequently ascribed to mumps are those of perivascular infiltration and proliferation. Despite the absence of apparent disease, these vascular changes were found in monkeys [3] and rabbits [2] at prolonged intervals after intracerebral inoculation of mumps virus and were associated with a mild meningeal reaction. Nerve cell damage was not prominent. Such late-appearing lesions are somewhat difficult to evaluate, particularly since the studies have not followed the gradual evolution of the lesions described and depend on studies of occasional animals killed at arbitrary intervals after inoculation.

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1. Donohue, W. L.: The Pathology of Mumps Encephalitis with Report of Fatal Case, J. Pediat. **19**:42, 1941.

2. deLavergne, V., and Kissel, P.: L'infection ourlienne, experimentale et humaine, Rev. immunol. **4**:411, 1938.

3. Johnson, C. D., and Goodpasture, E. W.: Experimental Immunity to the Virus of Mumps in Monkeys, Am. J. Hyg. **23**:329, 1936.

4. Wollstein, M.: An Experimental Study of Parotitis (Mumps), J. Exper. Med. **23**:353, 1916.

5. Van Rooyen, C. E., and Rhodes, A. J.: Virus Diseases of Man, New York, Thos. Nelson & Sons, 1948, p. 258.

6. Gordon, M. H.: Experimental Investigation in Relation to Mumps, Reports to the Local Government Board on Public Health and Medical Subjects, New Series, No. 96, Great Britain, 1914.

Fatal cases of human mumps encephalitis, as noted, are rare, but such descriptions as are available tend to stress the perivascular type of lesion.[1] However, even if one accepts the opinion that perivascular reactions are associated with mumps encephalitis, it does not necessarily follow that these lesions are a direct effect of the virus. In both humans and animals the late appearance of the vascular reactions has been emphasized, and it has been suggested that an allergic mechanism, presumably reaction of virus and antibody, may produce the vascular changes.[1] Thus it is apparent from the foregoing that neither the changes induced in the central nervous system by mumps virus nor the mode of action of the virus in producing tissue changes is satisfactorily established. Recently, propagation of mumps virus has been accomplished in brains of suckling mice[7] and hamsters,[8] and the fatal disease induced in these animals makes possible a more definitive experimental approach to the problem of mumps meningoencephalitis. Mumps virus produces an encephalitis in suckling hamsters which is evident grossly six or seven days after inoculation and which progresses to the death of the animal several days later. In mice, adapted strains of mumps virus cause a similar disease. The present paper describes in detail the histopathology of the meningoencephalitis induced in the brains of mice and hamsters and the evolution of these lesions as observed at various intervals following mumps virus inoculation.

MATERIALS AND METHODS

Virus.—Mumps Hamster Strain: This mumps virus was originally isolated from the milk of a patient having mumps at the time of delivery[9] and has been passed seven times in embryonated eggs by amniotic inoculation. The seventh egg passage was then inoculated into suckling hamsters intracerebrally and carried through 10 passages. The extracts used in the present experiments were prepared from the seventh and eighth suckling hamster brain passages. These extracts had an infectivity titer for eggs and hamsters of approximately log 5.0. The infectious agent recovered from the hamster brains at various passages was confirmed to be mumps virus by neutralization and hemagglutination-inhibition tests using known positive human mumps antisera.

Mumps Mouse Strain: The sixth passage suckling hamster brain extract was inoculated intracerebrally into one-day-old suckling mice and has been carried in mice for 23 consecutive passages. In this study, 15th passage material was used. This infectious agent was shown to be mumps virus by the tests mentioned above.

Preparation of Tissues for Histologic Examination.—Animals for histopathologic examination were killed by exsanguination. The skin and soft tissues were cut away to expose the calvarium, and, in the case of the hamsters, a window was cut in the calvarium to facilitate penetration of fixative. The whole heads were then removed and fixed in 10% aqueous formalin for a week or more. After fixation, heads were cut with a razor blade into three or more transverse slices 5 mm. thick. Slices were hardened for three days in 2.5% aqueous potassium bichromate, rinsed and decalcified in 5% aqueous formic acid, washed, and embedded in paraffin. The spinal cord was examined by dissecting the spinal column free of most of the soft parts, hardening and decalcifying, and cutting into four or more transverse blocks. This procedure gave undistorted preparations of meninges, nerve roots, and ganglia, as well as of the spinal cord. Cross sections of the head, in addition to displaying the brain, permitted satisfactory examination of the major salivary glands. Paraffin sections were stained routinely with azure-

7. Kilham, L., and Murphy, H. W.: Propagation of Mumps Virus in Suckling Mice and in Mouse Embryo Tissue Cultures, Proc. Soc. Exper. Biol. & Med. **80**:495, 1952.

8. Kilham, L., and Overman, J. R.: Natural Pathogenicity of Mumps Virus for Suckling Hamsters on Intracerebral Inoculation, J. Immunol. **70**:147, 1953.

9. Kilham, L.: Mumps Virus in Human Milk and in Milk of Infected Monkey, J. A. M. A. **146**:1231, 1951.

eosin and hematoxylin and eosin and in special instances with cresyl echt violet for Nissl substance and the Hortega-Foot silver impregnation for reticulum. Because of the delicate and incomplete myelination of these immature brains, preparations stained for myelin sheaths were not satisfactory for detecting demyelination.

EXPERIMENTAL

Histologic Lesions Induced by Mumps Virus in the Brains of Suckling and Adult Hamsters.—Suckling hamsters appeared to be "naturally" susceptible to infection with the mumps virus employed, in that virus extracts from human milk (or only a few egg passages removed) have induced in them a uniformly fatal encephalitis, whereas extracts far removed from the human failed to produce obvious disease.[8] In suckling hamsters, symptoms appeared eight to nine days after inoculation, and death follows in one to four days. The first experiments describe the gradual evolution of the mumps meningoencephalitis and attempt to correlate these lesions with the growth curve of mumps virus in the brains of suckling hamsters.

Comparison of Mumps Virus Growth and Development of Meningoencephalitis in Suckling Hamsters

	Days After Inoculation*				
	1	3	5	7	8 to 10
Average virus titer†	1.3	2.6	5.0	4.7	2.8
Histologic lesions	No examination	Minimal perivascular lesions confined to the midbrain	Marked perivascular lesions throughout the brain	Very early focal necrosis in areas of the most marked perivascular reactions	Frank necrosis scattered throughout the brain
Signs of illness	No signs of disease				Stiffness of limbs, prostration, incoordination

* Intracerebral inoculation of seventh suckling hamster brain extract (log 4.0 ID_{50} of mumps virus as determined in eggs).

† Titers represent egg infectious doses and are averages of two pools, each pool consisting of two hamster brains.

Six litters of suckling hamsters 2 to 4 days of age were inoculated intracerebrally with 0.02 ml. of a 10% extract of brains from the seventh suckling hamster passage. At intervals of one, three, five, seven, and nine days after inoculation, some of these animals were killed for determination of the virus growth curve, details of which have been described previously[8]; the remaining litter mates were killed at corresponding intervals for a study of the histologic changes induced by mumps virus at these stages of the infection. Some animals were sacrificed for histologic examination at 4, 6, and 10 days after inoculation, although virus titrations were not done at these intervals. As controls, two litters of suckling hamsters were inoculated with a 10% extract of normal hamster brain, and these animals were killed at intervals after inoculation, comparable with those in the experimental group.

RESULTS

Changes in Suckling Hamster Brain Induced by Mumps Virus.—The results of this experiment are summarized in the Table. After an initial fall in titer, virus multiplication began and was well established by the third day. Maximal growth of virus was attained by the fifth to the seventh day, and the titer thereafter declined.

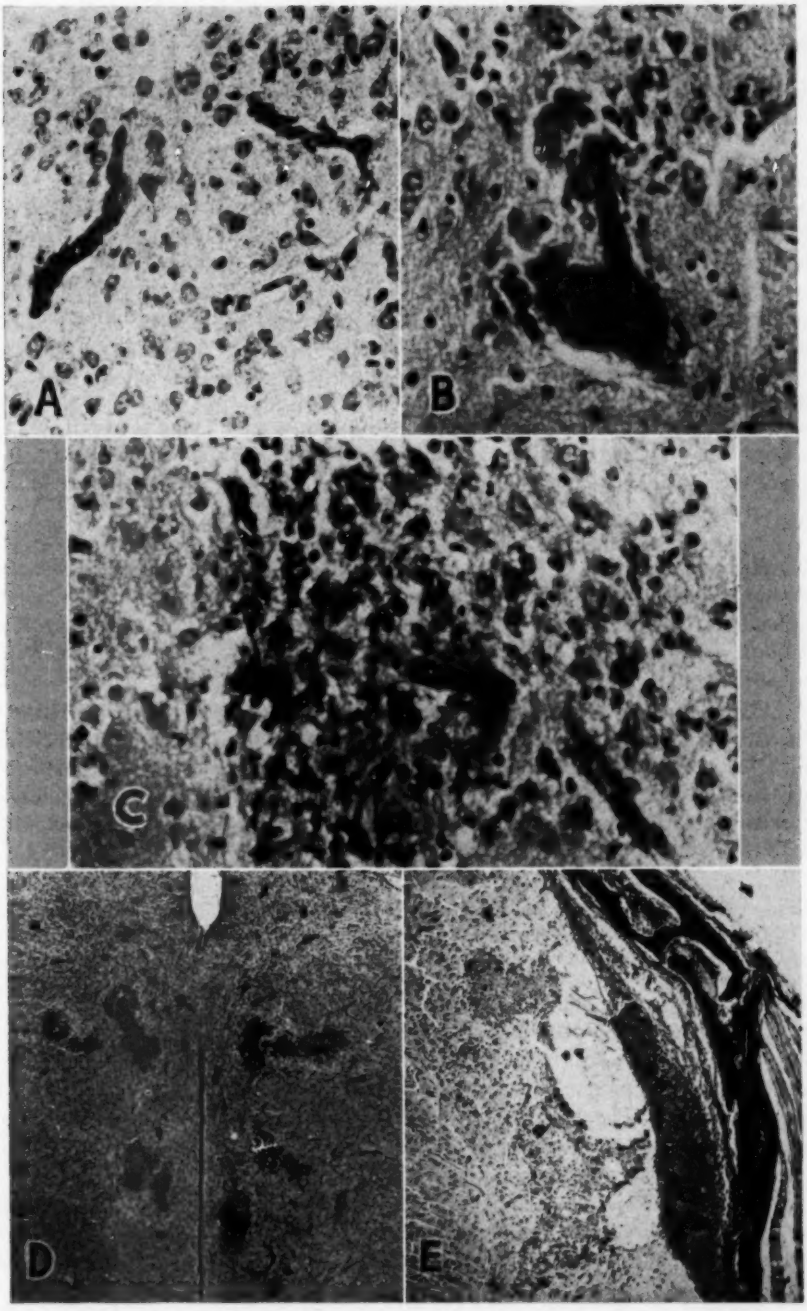
Gross changes of encephalitis in these brains were readily apparent. On the third or fourth day after inoculation, some hyperemia of the brain surface was evident in comparison with control animals. By 7 days, vascular congestion was

quite marked, and, with the onset of illness at 9 to 10 days, small discrete hemorrhages were present, particularly in the midbrain.

Microscopically, encephalitis caused by mumps virus in the suckling hamster was characterized by perivascular proliferation and infiltration in both white and gray matter, followed by inflammatory reaction and necrosis in the nervous parenchyma. This perivascular reaction appeared earliest and became most evident in the midbrain but soon spread peripherally to involve the basal ganglia, cerebral hemispheres, and brain stem. The cerebellum appeared to escape damage almost to the end, and in the final stages lesions in the cerebellum were limited to a few small perivascular infiltrations about vessels in the tectal white matter.

The earliest detectable microscopic lesion appeared on the third day after inoculation. It consisted of proliferation of cells forming the wall of a few of the small deep vessels in the midbrain (Fig., *A*). Normally such vessels appear as slender blood-filled tubes with almost invisible walls, along which are scattered oval nuclei, probably endothelial. In infected brains, these cells increased in number, although no mitoses were seen, to form an almost solid pavement of nuclei along the affected vessel. Simultaneously with the earliest vascular lesion, a patchy infiltration of small lymphocytes mingled with a few monocytes, and small numbers of red blood cells appeared in the roomier portions of the subarachnoid space. By the fourth day cellular proliferation in small intracerebral vessels was more prominent and widespread although still concentrated in the midbrain. At the same time small numbers of inflammatory cells, apparently lymphocytes with small dark nuclei and scarcely visible cytoplasm, began to appear in the brain parenchyma near affected vessels, and about some vessels there were microscopic extravasations of blood. By the fifth day cellular proliferation in vessels had progressed rapidly, and many vessels were marked by thick collars of cells. The origin of these cells could not be determined exactly, as even silver reticulum impregnations showed no definite perivascular space but only a loose and very delicate web of argyrophil fibers arranged longitudinally or in a loose spiral about vessels. The proliferating cells appeared among them, and, as they increased in number, the fibrils were lost to sight. At the height of this perivascular proliferation, many affected vessels appeared selectively crowded with polymorphonuclear leucocytes, but few, if any, migrated outside the vessel lumen. Other affected vessels were so compressed by the proliferating cells that their lumen became greatly constricted or entirely obliterated (Fig., *B*). No thrombus formation was observed.

Definite changes in the brain parenchyma were first visible on the seventh day, although a little earlier there was beginning activation of microglia evidenced by increase in number and deeper staining of these cells. The earliest lesion consisted of small ill-defined areas of necrosis located in the midbrain in the area of maximum vascular reaction (Fig., *C*). This necrosis involved all cells within the area, rather than selectively affecting one or more types of cells. Thus edema and partial liquefaction of the ground substance of the brain was accompanied by ameboid changes in the glia and degeneration of included nerve cells. The nerve cell nucleus appeared shrunken and fragmented, while the cytoplasm lost its scant chromidial substance and became somewhat shrunken and oxyphilic. Other nerve cells appeared to become vacuolated or frayed and then to liquefy and disappear. About some degenerating nerve cells there was a loose satellitosis of microglial cells, while about many others, which perhaps undergo more rapid degeneration, there was little or no glial reaction.



A, earliest lesion found in suckling hamsters inoculated intracerebrally with mumps virus. Endothelial proliferation in the vessel wall in the midbrain three days after inoculation. Van Gieson stain; × 330. *B*, seven days after inoculation the vascular infiltration and proliferation has proceeded to complete occlusion of the vessel in the suckling hamster brain. Van Gieson stain; × 330. *C*, early necrosis in the thalamus of a suckling hamster seven days after inoculation. Azure-eosin stain; × 330. *D*, bilaterally symmetrical necrosis in the thalamus of a suckling mouse 21 days after inoculation. This animal had shown marked signs of illness but was recovering at time it was killed. Van Gieson stain; × 38. *E*, combined necrotic and cystic lesion in the cortex of a suckling mouse killed 21 days after inoculation. Van Gieson stain; × 63.

No inclusion bodies were observed. From the 7th day to the 10th, when the experiment was terminated due to the imminent death of the remaining animals, the areas of partial necrosis of brain tissue increased slowly and irregularly in number and extent, spreading chiefly upward into the hippocampus and cerebral cortex.

The pathogenesis of these necrobiotic areas is not entirely clear. That they were not the primary lesion seems apparent from the fact that the vascular lesions were fully developed and widespread before the first necrosis of brain tissue appeared. Local ischemia caused by the marked degree of perivascular proliferation and subsequent vascular occlusion seems a more likely explanation. However, direct injury by virus may have played some part, as similar geographic areas of partial necrosis are a feature of other types of viral encephalitis in rodents not accompanied by so marked a perivascular reaction.[10] It is of interest to note that overt clinical illness in the animals did not appear until after the seventh day, although the perivascular reaction was fully developed, and that its onset coincided approximately with the appearance of frank necrosis of brain tissue. Simultaneously, the virus titer in the brain diminished sharply.

Except for the spinal cord, other organs of infected animals showed no significant abnormalities. In particular, the major salivary glands were well visualized and showed no lesions of any sort. Spinal cords showed irregularly minimal lesions of the same sort as those in the brain, but perivascular proliferation was scanty, and necrosis was limited to a few scarcely visible small patches. Brains and organs of animals injected with sterile brain extract as controls showed no abnormalities.

Lesions Induced by Mumps Virus in the Adult Hamster Brain.—Various attempts to propagate mumps virus in adult hamsters have so far been unsuccessful, and virus injected intracerebrally into adult animals can rarely be recovered seven days later. However, in view of the findings in suckling hamsters, it was of interest to examine the brains of adult hamsters at various intervals after inoculation with extracts of mumps infected suckling hamster brain.

Seven 21-day-old hamsters were inoculated intracerebrally with 0.03 ml. of the eighth suckling hamster brain passage material. These animals were killed at 7, 14, and 21 days after inoculation and the brains prepared for histologic study as described in the section on Materials and Methods.

Although the adult hamsters did not show any signs of illness in the course of the experiment, those animals killed at seven days did exhibit slight but definite gross evidence of encephalitis. Microscopically, both hamsters killed at seven days showed numerous perivascular infiltrations, with a few areas of microgliosis. Lesions were most prominent in the midbrain, but some were present in the anterior pons and medulla. The patchy round cell meningitis was also observed. At 14 days, only rare perivascular infiltrates were present, as was a scanty meningeal reaction. Three hamsters killed at 21 days showed no lesions.

These results would seem to indicate that some growth of virus does occur in the adult hamster, although passages in the adult were not accomplished. A similar situation was found by Kilham [11] in studies of Newcastle disease virus in adult and

10. Olitsky, P. K., and Casals, J., in Viral and Rickettsial Infections of Man, edited by T. M. Rivers, Philadelphia, J. B. Lippincott Company, 1948, p. 163.

11. Kilham, L.; Loomis, L. N., and Peers, J. H.: Variations in Behavior of 2 Strains of Newcastle Disease Virus on Passage Through Brains of Adult Mice, Am. J. Vet. Res. **13**:95, 1952.

suckling mice. Newcastle disease virus (California strain) grew readily and in high titer in suckling animals, but in adult mice, although striking and widespread lesions of a severe virus encephalitis were present, further passage of virus was not possible.

Studies of Mumps Virus Lesions in Adult and Suckling Mice.—Mumps virus has been propagated in the brains of suckling mice for 17 passages in this laboratory. However, in contrast to mumps infections in suckling hamsters, the incubation period is much prolonged (12 to 16 days), and about 20% of each litter inoculated survives the infection.[12] Moreover, either the virus has lost some of its ability to infect eggs or the titer of the brain extracts is simply low. Generally, suckling mouse brain extracts did not titer more than 10^{-1} in eggs, and thus a mumps virus growth curve for mice, similar to that for the hamster, was not feasible. The following experiment was designed to compare the histologic changes in mice induced by a mouse-adapted mumps virus with those in hamsters following injections of mumps hamster virus.

Fifteenth passage suckling mouse brain extract was inoculated intracerebrally into three litters of one-day-old suckling mice, and animals were killed for histologic study at 3, 4, 5, 6, 7, 9, 11, 16, and 21 days after inoculation. Three animals in this experiment survived, and at the 21-day period two mice which had been ill were entirely well; the other mouse was not completely recovered but undoubtedly would have survived. Six adult mice were inoculated with the same extract of suckling mouse brain mumps extract and were killed at 7, 14, and 21 days after inoculation. As in the hamster experiments, some animals were inoculated with normal mouse brain material to serve as controls.

In general, histologic changes described for hamsters were also present in infected mice. Thus perivascular proliferation and infiltration occurred early in the incubation period, and it was the predominant lesion found most abundantly in the midbrain but later spreading peripherally to involve most of the brain. Necrosis was again a late manifestation. In suckling mice, however, the appearance of the lesions lags several days behind those of the suckling hamster, which is in accord with the prolonged incubation period. Moreover, there was great irregularity regarding individual animals. With the hamster, all animals killed at the same interval after inoculation showed histologic changes of essentially the same type and degree of severity, whereas some mice would show extensive brain lesions, and others (litter mates) would show none at all. Presumably the latter group would have survived the infection. Of the three mice which showed signs of illness but which recovered and were well 21 days after inoculation, all showed perivascular infiltrations and scattered areas of gliosis. Two brains showed interesting symmetrical reactions consisting of granuloma-like areas of necrosis (Fig., *D*). These areas appeared more dense in consistency than the surrounding brain and showed no sign of the liquefaction generally present in terminal stages of brain necrosis. In these same animals, other combined necrotic and cystic lesions were present in the cerebral cortex (Fig., *E*).

Inoculated adult mice did not appear ill at any time, and gross evidence of encephalitis was not present. However, both mice killed seven days after inoculation showed a few perivascular lesions. Animals killed at 14 and 21 days after inoculation failed to show lesions of any type.

12. Recent passages (26th or more) produce uniformly fatal infections in all one-day-old mice inoculated intracerebrally with this mumps virus strain.

COMMENT

The foregoing studies clearly show that mumps meningoencephalitis is a distinct pathologic entity in mice and hamsters and that mumps virus can exhibit a neurotropism characterized by gross and microscopic changes in the brain and by death of the animal. This is of interest since mumps virus infections of the central nervous system in man and in experimental animals are generally benign, so much so, in fact, that the existence of an encephalitis directly due to mumps virus has been in doubt. The present experiments show that mumps virus can produce a severe meningoencephalitis and that it has definite neurotropic characteristics since, despite widespread changes in the brain, no histologic evidence of virus growth was found in the salivary glands or any other organs of the animal studied.

The most consistent lesion of mumps encephalitis in mice and hamsters is that of perivascular infiltration and proliferation. This reaction has been associated with mumps infections of the central nervous system and has been described in the human,[1] monkey [5] and rabbit.[2] In the latter instances, however, the perivascular reaction appeared to be a late development in the course of the disease, and some question might be raised as to whether the vascular lesion encountered is due directly to the effect of mumps virus or to some indirect mechanisms, such as reaction of virus and antibody, or to activation of some latent virus, presumably one which has more capacity to damage tissues than does mumps virus. In infected mice and hamsters there is no doubt that the perivascular reaction is a direct effect of mumps virus. It is the earliest lesion found, appearing within three days of inoculation and at a time when no detectable antibody levels are present.[13] There was no evidence that any latent virus complicated these studies. This, of course, does not exclude the possibility that one mechanism may cause the lesions in these rodents, whereas another may be operative in man or monkey; however, it at least shows that mumps virus per se has the potentiality for producing perivascular infiltration and proliferation.

Although the primary effect of mumps virus in the brains of suckling mice and hamsters is on vascular tissue, it is uncertain from the present experiments whether the virus may also affect nerve cells directly. As previously noted, nerve cell degeneration appears to occur both with and without a reactive glial response. This may mean that in areas where neuronal degeneration appears with satellitosis of glial cells but without necrosis the virus has had a direct effect on the nerve cell and that in the areas of focal necrosis without gliosis the nerve cell degeneration is due to ischemia caused by vascular occlusion.

The immaturity of some elements in the newborn brain makes obscure certain features of the mumps histopathology. The differentiation between arteries and veins in the brains of suckling animals is difficult, so that the perivascular lesions may be either periarterial or perivenous. However, no perivascular reaction has been found around those vessels which could be classified as veins, so that in all probability the changes are associated with arteries or capillaries. Myelin is not present in these young brains in any detectable quantity, and therefore the presence or absence of perivascular demyelination could not be determined. The nerve cells,

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for the most part, consist of a large nucleus with a small scanty cytoplasm and little or no chromidial substance, so that chromatolysis and cytoplasmic changes are not readily apparent.

The relationship between the primary mumps reaction of perivascular infiltration and proliferation and the signs of disease and virus growth is of interest. Thus it would appear that the vascular lesion may be quite marked without causing obvious illness of the animal. Suckling mice and hamsters appear entirely well at a time when the perivascular reaction is quite extensive. Likewise, the presence of widespread perivascular lesions in the adult hamsters does not seem to cause illness. In suckling hamsters the virus titer is quite high during the development of the vascular reaction and drops when necrosis occurs, yet in the adult hamster, although similar vascular changes are present, no virus growth has been detected.[13] Mumps virus infections of embryonated eggs yield the infecting agent in high titer from tissues in which pathologic alteration is remarkably scanty.[14] One strain of Newcastle disease virus in mice, however, has produced results similar to those encountered in the present experiments.[11] The California strain of Newcastle disease virus, when injected into suckling mice, produces a severe encephalitis, and the virus can be recovered in large amounts. When this strain is injected intracerebrally into adult mice, the histologic alterations are similar to those in the suckling mice, but no virus can be recovered after injection of an extract of these brains into either eggs or suckling mice. Here, then, are examples of viruses which produce tissue changes suggestive of virus growth, but virus multiplication is not detectable by conventional methods. Various explanations might be offered, but all are highly speculative. Thus, some "toxic" effect of mumps virus may have produced this effect, although this does not seem likely, or the phenomonon described may be a manifestation of a so-called "incomplete" virus.[15] Certainly it is difficult to understand how a virus which does not multiply could produce widespread cellular changes morphologically identical to those found when definite growth of virus can be readily demonstrated.

SUMMARY

Mumps virus propagated in brains of suckling mice and hamsters produces a severe gross and microscopic meningoencephalitis. The earliest lesion is that of perivascular proliferation and infiltration, initially found in the midbrain but later spreading peripherally to involve most of the gray and white matter of the brain. In many instances the vascular lesion proceeds to complete occlusion of blood vessels, and signs of ischemic necrosis appear. In a few areas nerve cell degeneration appeared to be due to a direct destructive effect of mumps virus. In relation to the growth curve of the virus in suckling hamsters, the virus titers appear to increase as vascular lesions progress. With the onset of necrosis in the brain substance, however, the virus titers fall. The strains of mumps virus used in the present experiments did not produce illness in adult mice or hamsters, and the virus could not be passed in them. Extracts of infected suckling hamster brains did, however, produce in adult hamsters extensive vascular lesions located predominately in the midbrain but without necrosis of nerve cells or of brain substance.

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EXPERIMENTAL INDUCTION OF IRON OVERLOAD IN THE RAT

I. Morphological Alterations Due to Dietary Siderosis

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IN THE past 10 years, through the efforts of Granick,[1] McCance,[2] Hegsted,[3] and other investigators,[4] radical changes in our concepts of iron metabolism have evolved. The mechanism of iron storage represents one link in the biologic cycle of iron metabolism which has been strengthened. The apparent inability of the body to excrete iron,[5] the avid binding properties of proteins for that metal,[6] and the behavior of the gut epithelium offering a "mucosal control" over iron absorption are all newly discovered factors intimately concerned in iron storage and accumulation.

As to the morphologic alterations of excessive iron storage, observations on the developmental pattern of disease have been derived from several fields. The pathologic anatomy of "exogenic hemochromatosis" following multiple blood transfusions has been recorded from autopsy studies,[7] and experimental studies have shown that rats on a low protein-phosphate diet with added iron salts stored excessive amounts of iron within their depot tissues.[4]

This investigation into iron overload represents a confirmation and extension of Hegsted [3] and Kinney's [4] work on iron accumulation which developed in rats maintained on a corn grit-iron salt diet.

Our experimental studies were carried out for a greater length of time and in combination with other forms of tissue injury in an attempt to induce not only tissue siderosis but other concomitant alterations which more closely parallel those seen in idiopathic hemochromatosis.

MATERIALS AND METHODS

Fifty-five young male white rats weighing between 117 gm. and 63 gm., with an average of 85 gm., were divided into two groups. Group A received diets [8] as follows: 10 rats, corn grits, lard, and 2% powdered ferric citrate; 10 rats, ground Purina chow and 2% ferric citrate; 5 rats, corn grits and lard; 5 rats, Purina chow. Group B was subjected to bilateral orchiectomy and

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6. Schade, A. L.; Reinhart, R. W., and Levy, H.: Arch. Biochem. **20**:170, 1949.
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8. Diet composed of corn grits obtained from Lawhoff Grain Company, South St. Louis, and lard from Swift Packing Company.

received diets as follows: 10 rats, corn grits, lard, and 2% ferric citrate; 5 rats, corn grits and lard; 5 rats, Purina chow and 2% iron citrate; 5 rats, Purina chow. After 72 days, the diets containing iron in half the rats in Group A and B were further supplemented with 0.5% powdered copper acetate. In both groups, the specific diets and water were provided ad libitum.

The animals fed corn grit in both groups, regardless of the addition of iron or castration, soon lost their usual sleekness, developed ruffled coats, and eventually became grossly emaciated, many losing up to 20 gm. in weight. The rats fed chow and iron citrate gained weight but not as rapidly as those fed chow alone. When copper acetate was added to the corn grit-iron diet, further deterioration in the condition of the animals became obvious, and death occurred after several weeks. The lowest hemoglobin recorded during the experiment was 15 gm.

As the rats died or were killed, their tissues were fixed in 10% formalin solution and sections of the liver, spleen, pancreas, and kidneys were examined. Stains used included hematoxylin and eosin, Turnbull and Perl's staining reaction, and, in selected instances, Mallory's trichrome connective tissue stain and Bourne's silver nitrate stain for reduced ascorbic acid.

RESULTS

The results are expressed in tabulated form (Table).

Morphological Evaluation of Tissue Siderosis *

No. of Days on Diet	Liver			Pancreas		
	Corn Grits, Iron	Normal Chow, Iron	Corn Grits, Iron, Castration	Corn Grits, Iron	Normal Chow, Iron	Corn Grits, Iron, Castration
1 to 50	+	—	+	No death	—	—
	No death					
50 to 100	++	No death	+	—	No death	—
			No death			
100 to 120	+++	No death	++	—	No death	—
120 to 150	+++	—	+++	—	—	—

* +, iron present, periportal; ++, maximal deposition of iron at periphery of lobule; +++, severe uniform distribution of iron; —, no iron; no death, no animal died in the time period.

Liver.—The liver in Group A rats (fed corn grits and 2% ferric citrate) at 72 days showed maximal deposition of iron at the periphery of the lobule. Small numbers of liver cells contained iron granules, but the main bulk of the iron was deposited in the Kupffer cells lining the sinusoids. These cells were especially prominent in the region of the portal tracts where they formed small giant cells. The histiocytes of the portal tracts also contained iron granules.

These changes were observed in the liver up to 128 days; the iron overload became greater; the liver cells in all parts of the lobules took the Prussian blue stain; the Kupffer cells became larger, showing more iron granulosity, and coalescing to form large giant cells. The latter gradually came to occupy all parts of the lobules. Although the histiocytes of the portal tracts showed a concomitant increase in iron no evidence of cirrhosis in any animal was found.

After 72 days, 0.5% copper acetate was added to the corn grit-iron diet. Seventeen days following this, the appearance of the liver differed in no remarkable way from that previously described, except that the iron uptake was slightly greater. After 32 days of copper and 100 days of iron, the giant cells, while still preserving this affinity for the portal tracts, were scattered throughout the lobule and appeared to be completely blocking the sinusoids, but no associated central necrosis of the lobule was observed. Again, although the histiocytes of the portal tracts contained iron, no increase in fibrous tissue was found.

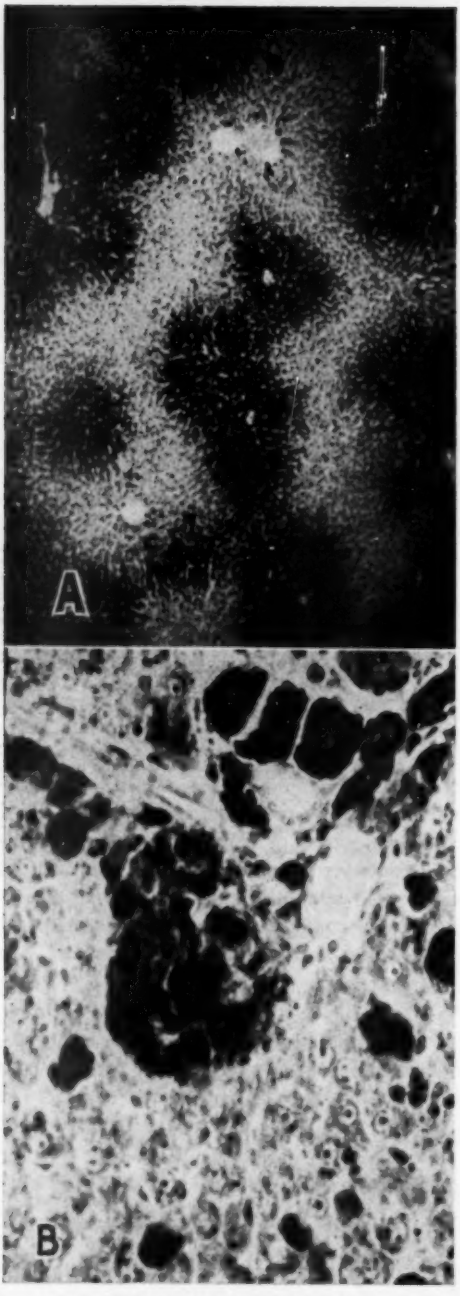

Fig. 1.—*A*, diffuse concentration of hemosiderin in the peripheral zones of lobules in rat liver after 35 days of corn grit-2% ferric citrate diet. Prussian blue; × 40. *B*, heavy accumulation of iron in giant Kupffer cells in sinusoids of outer third of liver lobule in rat fed corn grits-2% ferric citrate for 78 days. Prussian blue; × 320.

The giant cells described above, when seen in the hematoxylin and eosin sections, appeared as large, pale-staining cells without a visible nucleus, the cytoplasm being filled with yellowish-brown granules which gave the cell its distinctive color. When stained with Prussian blue, the cells became an intense mandarin black color without much granularity, and again no nucleus was seen.

In the castrated Group B rats, fed corn grits and 2% ferric citrate, the first liver was examined at 34 days. The iron was contained solely in the Kupffer cells of the peripheral part of the lobule, the liver cells and histiocytes of the portal tracts being entirely free. No giant cells were observed. At 74 days and up to 120 days, the liver changes corresponded with those in Group A at a similar period. No differences were observed, except that the iron uptake was not quite so intense.

Nineteen days following the addition of 0.5% copper acetate, the liver again resembled that of Group A rats at a similar period. After 64 days of copper acetate, the last rat in this group was killed. The iron accumulation was intense within the liver and was of the same distribution as previously described. Huge giant cells were present throughout the lobule; all the liver cells contained iron granules, and the histiocytes of the portal tracts were loaded, but again no cirrhosis was seen.

When the results obtained above were compared with the results obtained in the chow-2% iron-fed animals, a striking difference was noted. Even after 80 days on the iron diet, no hemosiderin was demonstrated in the liver. The addition of 0.5% copper acetate made little difference because, even when the experiment was terminated after 150 days of iron and 80 days of copper, the liver iron was confined to minute amounts in the Kupffer cells at the periphery of the lobule, all other areas being free.

Spleen.—The spleen of the iron- and copper-fed animals in both groups showed an increase in iron which became apparent slightly before the liver pigmentation. This was especially noticeable in the animals on normal chow-2% ferric citrate diet. Neither the addition of copper to the diet nor castration made any difference in the iron uptake of this organ. The iron was found in the macrophages of the parenchyma and, although distributed throughout the pulp, showed a tendency to encircle the Malpighian bodies and demarcate the inner core of Flemming's germinal center from the peripheral lymphocytic border. The latter regions remained free of iron for a long time, but eventually minimal deposition was observed in the corn grit-iron-fed animals, with and without castration. The iron containing macrophages formed giant cells similar to those in the liver, although slightly smaller; in no instance was the iron overload so intense as that observed in the liver. Abdominal lymph nodes examined showed the same reaction as the spleen. The control animals fed normal chow, corn grits, or chow and copper did not reveal iron accumulations within the liver or pancreas.

Kidney.—In all animals fed corn grits and iron, regardless of castration or time of death, an occasional proximal convoluted tubule cell which stained slightly with hydrochloric acid and potassium ferrocyanide (Perl's) was seen. However, all other parts of the kidney were free.

Two rats fed corn grits and iron, one each in Group A and B, were found to have a focal pyelonephritis. In the scar tissue of the kidney, histiocytes staining intensely for iron were observed. In addition, many proximal convoluted tubules closely related to the areas of scar tissue were found to contain fairly heavy iron deposits.

PATHWAY OF IRON METABOLISM

The absorption, transport, utilization, and storage of iron represents one of the more precise regulatory mechanisms in mineral metabolism. From previous investigations, it is apparent that iron is converted, owing to the acid pH of the stomach, to the soluble ferrous state, and in this form it is absorbed by the mucosal cells of the duodenum. Granick [9] and other investigators [10] have shown that ferrous iron within the cell is oxidized to ferric iron and combines with a specific protein, apoferritin, to form the iron hydroxide-protein complex ferritin. The latter complex releases the iron which passes into the plasma, and the mineral is combined with a specific globulin, siderophilin, and in this form is transported to the liver and other depots and once again stored as ferritin. There is no evidence as yet to suggest that, under dietary experimental conditions which held in this experiment, any alterations other than plasma saturation occurred in the carriage of iron.

McCance and Widdowson [2] have shown that iron, once absorbed, cannot be excreted; the passage of iron through the mucosal cell is in one direction only. Further, Hahn [11] has shown by radioactive iron studies that a "mucosal barrier" exists, limiting iron absorption and thereby preventing tissue saturation. But if the "mucosal barrier" is seduced, the ferritin content of the storage depots is increased at a maximal rate, and more and more iron in an unnatural state is deposited on the ferritin micelles, eventually resulting in microscopically visible granules of hemosiderin.

Excessive storage of iron in the tissue depots indicates that either the mucosal barrier has been bypassed by intravenous iron or its functional capacity is altered owing to protracted infusions, protein deficiency, or an imbalance of the iron-phosphate mineral ratio. From the Table it is noted that the storage of iron increases in direct proportion to the length of time during which the corn grit-iron diet is absorbed. From this experiment it is probable that both factors, protein deficiency and iron-phosphate imbalance, have been profoundly altered, leading to pathological excess of iron.

DISTRIBUTION OF IRON IN DIETARY SIDEROSIS

It is of value to compare the iron content and related changes in liver, spleen, pancreas, and kidney in dietary siderosis and those lesions described by Sheldon [12] in the same organs in idiopathic hemochromatosis.

The iron storage in the animal tissue was found almost exclusively within the reticuloendothelial system in the early phase, the most rapid accumulation of iron being within Kupffer cells. Subsequent to overload of the Kupffer cells, the iron accumulates in the hepatic cells of the periportal zone. A simple and usual explanation for this marginal storage of iron is that the blood supply enters the lobule from the periphery and that the cells take up iron on the basis of first come, first served. On the other hand, it has been postulated that the zone distribution of lesions in pathological states is related to distinctive zonal physiology. Noël,[13] through obser-

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vations on mitochondrial activity, and Seneviratne,[14] utilizing a transillumination technique on the liver, support the concept that the liver lobule possesses different zones of functional activity. The initial iron storage in the peripheral zone of the liver in the experimental animal or the rapid storage of iron in hemolytic and deficiency anemias in this region of the liver lobule, with reassimilation on clinical remission, suggests a functional zonal avidity for freshly liberated hemoglobin or ingested iron.

Although Nissim[15] has suggested a relationship between fixation of iron and ascorbic acid in the tissues, in our hands, Bourne's histochemical maneuver for reduced vitamin C did not reveal a distribution related to the hemosiderin storage pattern of the liver lobule.

From our animal experiments it is apparent that, as the iron overload within the liver increases, the Kupffer cells undergo coalescence to form giant cells (Fig. 2*A*). Most of these were heavily laden with iron granules; others showed light sprinkling. This finding confirmed the observation of Cappell[16] and, later, of Wyatt[7] that there is a constant regeneration of Kupffer cells. In the later phase of the experiment, the giant cells observed were present in all parts of the lobule and were of enormous size (Fig. 2*A*), blocking the sinusoids and causing some distortion of liver cords in their vicinity. Himsworth[17] has implied that sinusoidal blockage with consequent interference with the blood supply may result in centrilobular necrosis of the lobule followed by fibrosis. We have been unable to confirm this observation in iron overload.

As to the spleen, the pigment was present mainly in the macrophages and tended to form giant cells. Sheldon does not mention giant cells in hemochromatosis. But as in idiopathic hemochromatosis, the iron content of the spleen in dietary siderosis was much less than of the liver, despite the huge reticuloendothelial cellular component of the spleen.

As to the kidney and pancreas, there was little or no pigmentation. One observation of a minor nature was the concentration of iron in regions of inflammation. This was particularly noticeable in two examples of pyelonephritis, a common kidney disease in old rats which was encountered during the course of the experiment. Concentrations of iron were noted in the irregular scarred areas of kidney. Nissim[15] has indicated that in inflammatory-reparative processes, owing to the local elevation of vitamin C, there is increased iron storage. Examination of both pancreatic acinar tissue and islets of Langerhans failed to reveal any evidence of iron accumulation. This, to a certain extent, supports Gillman's[18] observation on the lack of pigmentation in the pancreatic islets in the South African pellagrin, in marked contrast to the heavy accumulation of iron in the liver parenchymal cells.

RELATIONSHIP BETWEEN DIETARY DEFICIENCIES AND IRON DEPOSITION

Examination of the livers of those "unfortunates" incarcerated and dying in the notorious Buchenwald and Belsen concentration camps showed hemosiderosis. This has been related to the low protein diet with consequent increased absorption

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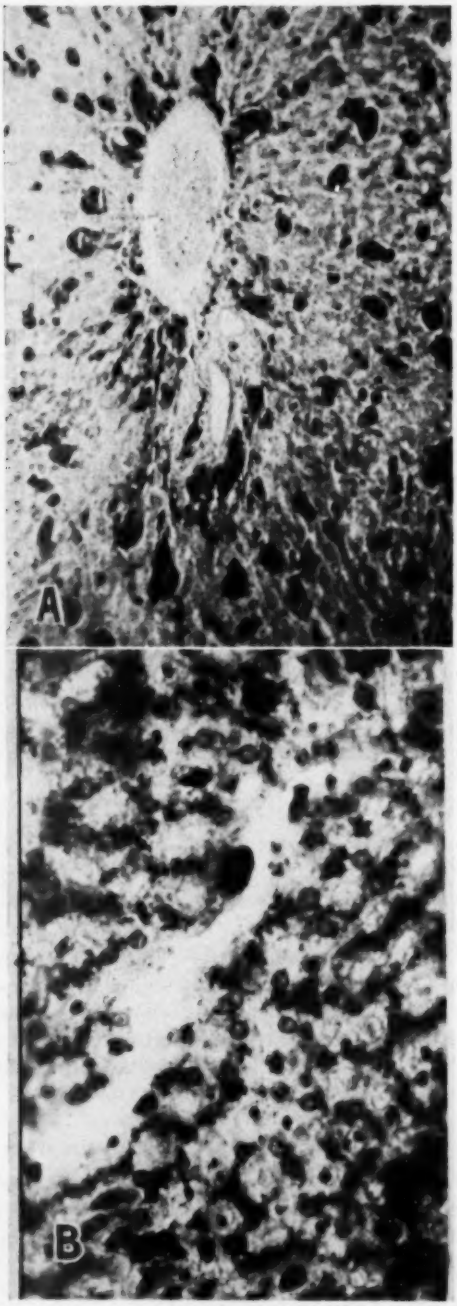

Fig. 2.—*A*, concentration of iron in parenchymal and reticuloendothelial cells in rat liver after 90 days of protein poor-iron rich diet. Prussian blue; × 180. *B*, diffuse concentration of iron universally distributed throughout rat liver lobule after 2% ferric citrate-corn grit diet for 130 days.

of iron or to emaciation with generalized reduction in body tissues and consequent storage of related tissue iron. Experimentally, it has been recorded that pyridoxine-deficient animals [19] showed moderate tissue siderosis.

It may be claimed that a combination of these factors was responsible for the iron accumulation observed in the experimental rat. However, in no instance did the control animals or those maintained on corn grit alone show any demonstrable iron within the liver. Furthermore, deposits were found in the liver within 7 to 10 days of the start of the experiment, leaving insufficient time for a dietary deficiency to manifest tissue alterations of iron overload. Heavy vitamin supplements to the corn grit diet did not suppress iron storage.[20]

These animal experiments have a counterpart in the work of Gillman and Gillman in their study of South African pellagrins who existed on a diet consisting mainly of corn maize cooked in iron pots. By means of aspiration biopsy and autopsy studies they obtained a sequential study of the liver in these natives. The liver lesions progressed from fatty metamorphosis to severe iron storage and in many instances terminated with structural alterations identical with idiopathic hemochromatosis. However, they pointed out that the amount of iron in the liver bore no relationship to the presence or absence of fibrosis. Furthermore, the initial localization of the iron within the liver differed from any previously described distribution in that it appeared first in the liver cells of the central part of the lobule, gradually progressed towards the periportal areas, and increased in intensity. Only in the later stages of the disease condition was the iron found in the Kupffer cells. From these observations, it would appear that there are a number of differences between cytosiderosis in the South African Bantu, classical hemochromatosis, and dietary siderosis (ferrichromatosis, Kinney's term [4]).

ROLE OF COPPER AND STEROIDS IN IRON METABOLISM

In 1921 and subsequent years, Mallory and others [21] claimed to have produced experimental hemochromatosis by prolonged copper feeding experiments. Hall and MacKay [22] were unable to confirm Mallory's results. In view of these conflicting opinions and results, the effect of 0.5% copper acetate mixed with a corn grit-2% ferric citrate diet was observed on animals already suffering from iron overload of the liver. These animals were under observation for a period up to 72 days. Despite the addition of copper to the diet, no difference in the iron storage pattern was noted, and there was no evidence of progressive fibrosis.

An apparent explanation for the increased copper content in chemically analyzed specimens from fatal cases of conventional hemochromatosis may be related to the absorption and the plasma carriage of copper. It is known that the intestinal canal absorbs only 25% of the oral copper and slowly excretes over a period of weeks any excess through the canal. With the knowledge that siderophilin is an essential for the transport of copper, as well as iron, it is interesting to speculate that under certain conditions the intestinal absorption is increased, tissue affinity for copper increases, and, with copper excretion continuing at a low level, storage of large amounts of copper occurs.

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Castration was incorporated into our experiment, based upon several considerations. As Sheldon has pointed out, idiopathic hemochromatosis is a disease occurring overwhelmingly in males, and testicular atrophy, with loss of libido associated with other evidence of sexual hypoplasia, frequently precedes the full-blown clinical manifestations of the disease; this finding is suggestive that endocrine disturbances may antedate the development of iron overload. Nissim has previously commented on the possible relationship between steroid distribution in tissue and iron localization. Furthermore, it has also been shown experimentally that injections of testosterone cause a phosphorus retention. While well aware that the converse might not be true, it seemed of value to test the possible effect of androgen withdrawal due to castration and its influence upon phosphate-iron control. From the Table it can be seen that we were unable to demonstrate a relationship on morphological grounds between iron storage and castration. The iron uptake and amount of iron present differed in no way from that of the other noncastrated animals.

RELATIONSHIP OF IRON ACCUMULATION TO CIRRHOSIS

Granick has postulated that one of the possible mechanisms of fibrous tissue production in hemochromatosis may be due to the intracellular precipitation of protein or inhibition of enzyme activity caused by iron in an unnatural state, with consequent cell death and replacement fibrosis. In the end stages of our experiment, all the liver cells were overcharged with iron granules, yet markings of cirrhosis were not present. Both Cappell [16] and Polson,[23] despite repeated intravenous injections of iron into small experimental animals, failed to induce pathological changes of hemochromatosis and concluded that "it is unlikely that excess of iron is responsible for hepatic and pancreatic lesions of human hemochromatosis." From our own observations, coupled with those of Kinney, we do not believe that iron per se in animal feeding will cause cirrhosis. Other observations confirming this opinion on the relatively inert nature of the iron are those of Cillman and others. These investigators injected the iron pigment obtained from the liver samples of South African pellagrins into cats and failed to obtain cirrhosis in this animal.

It would appear, then, that some factor (or factors) other than excess of iron is required for the development of progressive fibrosis in the rat on a high iron-low protein diet.

SUMMARY

In a series of rats fed iron salts with a corn grit (low protein) diet, excessive iron storage in the liver was produced. Although the experiment was carried out for 150 days, no pigmentary cirrhosis was observed. The effects of castration and ingestion of copper salts in animals developing tissue siderosis were also studied, but no evidence of progressive parenchymal fibrosis was observed. From these experiments on dietary siderosis of the rat, it would appear that some additional factor other than copper salts or androgen withdrawal is required to produce progressive pigmentary cirrhosis.

The relationship of these experimental findings is integrated with the whole problem of hemochromatosis.

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VASCULARITY OF THE EARLY SUBCUTANEOUS NODULE OF RHEUMATOID ARTHRITIS

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THE SUBCUTANEOUS nodule of rheumatoid arthritis presents a histological appearance that is well known to pathologists. Splendid descriptions of these have been written by Dawson,[1] Collins,[2] and Bennett and others.[3] Nevertheless, certain aspects of the pathogenesis of the lesion remain quite controversial. In the fully developed nodule, three zones are distinguished: (*a*) a central area of necrosis, (*b*) bordering on this a palisade of radially arranged elongated cells that have one or more nuclei, and (*c*) an external capsule of scar tissue. Within the latter, some proliferation of blood vessels may be seen. Usually this is not a conspicuous feature. Indeed, Collins has been so impressed by the paucity of vessels that he has drawn two important conclusions from this observation: The avascularity of the newly formed connective tissue (*a*) is related to the development of the central necrosis, and (*b*) is a morphologic characteristic distinguishing the rheumatoid nodule from that of rheumatic fever.

Nevertheless, a similarity in the character of the vascularity of the peripheral zone in both types of nodules has been observed by Bennett and by Dawson. Inflammatory changes in these vessels have been recognized in infrequent instances.[4]

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4. Keil, H.: The Rheumatic Subcutaneous Nodules and Simulating Lesions, Medicine **17**:261-380, 1938. Collins, D. H.: Observations on the Pathology of Acute Rheumatism and Rheumatoid Arthritis, Ann. Rheumat. Dis. **1**:38-45, 1939. Footnotes 1, 2, and 3.

It has been suggested that these resemble the peripheral arteritic lesions of rheumatic fever described by Von Glahn and Pappenheimer.[5]

The present study has disclosed that, unlike the indolent mature nodule of rheumatoid arthritis in which large amounts of necrotic detritus are the dominant component, the young nodule is a highly vascular structure. It is the purpose of this communication to describe certain peculiarities of this vascularity.

It is, of course, most difficult to ascertain the precise age of the early rheumatoid nodule. Its onset is insidious, and the patient frequently becomes aware of the presence of the nodule only after it has reached considerable proportion. In several instances the nodules observed in the present series were discovered only after a systematic search for incipient nodules had been made. In some instances they were found as the result of making serial sections of the suspected area. Aside from their small dimension, their youthfulness is attested to by their cellularity and by the minor extent and coagulative character of the necrosis.

REPORT OF CASES

Case 1.—M. Y., a Negro woman aged 33, complained of migrating polyarthritis three weeks after delivering her second child. The patient stated that she had had an attack of "rheumatism" at the age of 9 years and had been confined to bed for three months. She was admitted to the hospital on the eighth day of the present illness. At that time there were pain, swelling, and tenderness in several proximal interphalangeal and metacarpophalangeal joints of the hands; in the joints of the shoulders, the right elbow, the knees, the ankles, and in the metatarsophalangeal joints of the feet. The temporomandibular and hip joints were painful, stiff, and tender. Ten days following the onset of arthritis, one of us observed an early subcutaneous nodule over the free border of each ulna, about 2 in. (5 cm.) distal to the olecranon process. Biopsy of this nodule was performed 11 days later. Other positive physical findings consisted of generalized enlargement of the superficial lymph nodes. The liver and spleen were not palpable. The heart was normal. The erythrocyte sedimentation rate (Westergren) was 77 mm. per hour. The serum agglutination test for sensitized sheep erythrocytes was positive. Roentgenograms of the involved joints disclosed no destructive changes of the articular cartilages or the subchondral bone. The patient received neither gold salts nor adrenocortical hormones before biopsy was performed. Acetylsalicylic acid (aspirin) had been administered for five days and aminopyrine for two days immediately prior to excision of the nodule.

Subcutaneous Nodule (Q47).—The gross specimen consisted of two pieces of fibroadipose tissue measuring up to 3 cm. in greatest dimension. Serial sections revealed these to incorporate six or seven nodular lesions, individually measuring approximately 2 to 3 mm. in diameter. In one instance the nodule was larger, being 8 by 3 by 3 mm. in dimensions. Most of the nodules proved to be nests of granulation tissue. They were composed principally of tortuous, newly proliferated capillaries. In places only sparse amounts of loose fibrous tissue intervened between these vessels and small numbers of chronic inflammatory cells (lymphocytes, mononuclear cells, and plasma cells). A rare eosinophile was present. There were many Russell fuchsin bodies. These nodules superficially resembled hemangiomata. However, at the periphery of some of these, another component was seen: a zone consisting of intensely eosinophilic, refractive, interlacing fibers that merged imperceptibly with normal-appearing collagenous fibers within the vascular nodule. This material was disposed radially at the margin of the granulation tissue and then was whorled

5. Von Glahn, W. C., and Pappenheimer, A. M.: Specific Lesions of Peripheral Blood Vessels in Rheumatism, Am. J. Path. **2**:235-249, 1926.

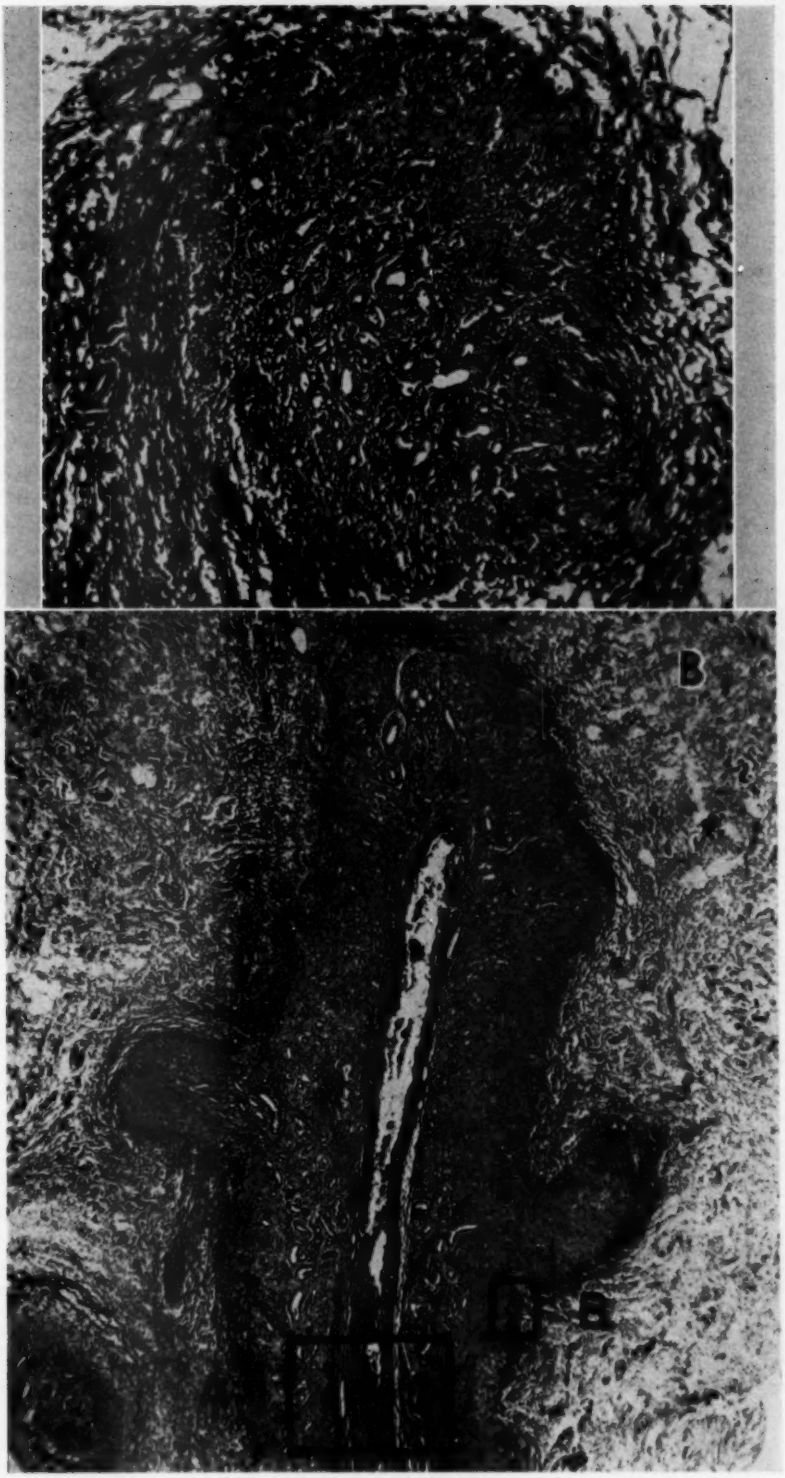

Fig. 1 (Case 1).—*A*, the darkly stained, coarse fibrous tissue surrounding the nodular core of granulation tissue is undergoing necrosis. The process appears to stream from the center as though fluid-borne into tissue planes. *B*, granulation tissue proliferating about small artery. The darkly stained border is a zone of necrosis. The adjacent fibrous tissue is edematous; × 11.

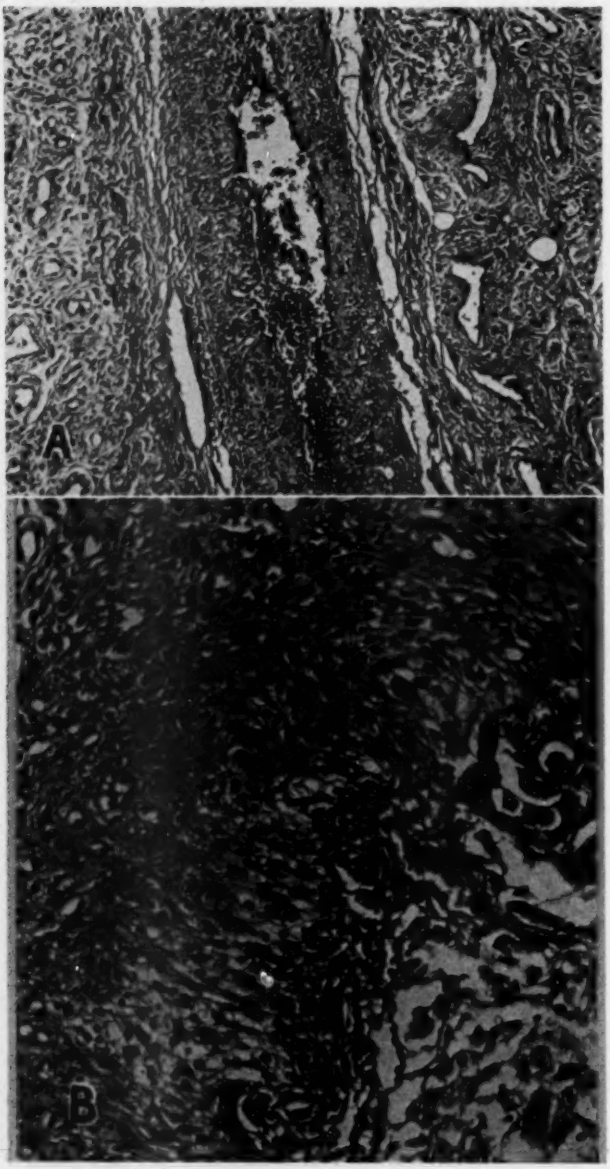

Fig. 2 (Case 1).—*A*, a higher magnification (× 46) of the framed area A in Figure 1 *B*. Vascular granulation tissue has proliferated about the artery; capillaries proceed from the lumen of the artery into its wall. A small area of hemorrhagic necrosis is seen in the intima. *B*, a higher magnification (× 220) of framed area (B), Figure 1 *B*. At the periphery of the granulation tissue is a palisade of radially disposed elongated cells and a zone of necrosis.

somewhat concentrically about it. The process thus appeared to stream from the vascular center, as though fluid-borne, into preformed fibrous planes (Fig. 1*A*). Gram and phosphotungstic acid-hematoxylin stains revealed these to stain like fibrin. A small number of elongated and fusiform mononuclear cells were oriented in a palisade at the junction of the normal fibers with the zone of "fibrinoid" change. The fibrous tissue adjacent to the nodules was somewhat edematous. In the center of the largest of the nodules of granulation tissue was a small artery, 75 μ in external diameter (Fig. 1*B*). The granulation tissue not only surrounded this artery but in most areas blended with its adventitia. Indeed, the vascularization involved part of the media and intima as well (Fig. 2*A*). As a result, the internal elastic lamella was disrupted at several points. A minute verrucous focus of necrosis was present in the intima. Fibrinous material, a few red cells, and some nuclear detritus were present here. Only at the periphery of the nodule were a palisade of radially arranged cells and a zone of necrosis seen (Fig. 2*B*). Some amorphous basophilic substance, presumably of nuclear origin, smudged parts of the necrotic material. Once again, the adjacent fibrous tissue was somewhat edematous.

CASE 2.—M. L., a 56-year-old white woman, was first seen in the arthritis clinic in September, 1951. The first attack of arthritis occurred in 1945, when, following a fall, pain developed in the right knee. The pain persisted for several months. A year later there was a recurrence of arthritis, with involvement of both knees. This episode also lasted for several months and subsided spontaneously. Recrudescences followed at intervals of several weeks; these lasted one or two days. The shoulders, wrists, and fingers were involved. Since March, 1951, the pain and stiffness of the shoulders were unremitting. Examination disclosed swelling, tenderness, and stiffness in several proximal interphalangeal and metacarpophalangeal joints of the hands; in joints of the wrists, knees, and ankles, and in metatarsophalangeal joints of feet. The shoulders were stiff and painful on motion. Subcutaneous nodules were present on the free border of each ulna near the olecranon process. The serum agglutination tests for hemolytic streptococci and for sensitized sheep red cells were both positive. The antistreptolysin O titer was normal (80 units per cubic centimeter). Roentgenograms of the affected joints revealed osteoporosis of adjacent bones but no destruction of the cartilage or bone. Chrysotherapy was initiated but abandoned after 100 mg. of gold salts had been administered because it was decided to employ adrenocortical hormones at that time. A preliminary course of intramuscular treatment with corticotropin, 100 mg. daily for six days, was followed by daily oral administration of cortisone. Cortisone acetate was first given on Nov. 11, 1951. The daily dose until the last biopsy was performed (June 25, 1952, 227th day) ranged from 100 to 62.5 mg. On Jan. 10, 1952, while the patient was being maintained on cortisone, new, small subcutaneous nodules, 2 to 3 mm. in diameter, appeared on the lateral border of several terminal phalanges and on the surface of the palms. Two of these were removed from the fingers for histological examination. In addition, another nodule was excised from the ulnar region from which a previous nodule had been removed some half year before. The latter nodule was, therefore, less than 6 months of age. The clinical condition of the patient improved initially but thereafter was little affected. The erythrocyte sedimentation rate remained elevated. The patient had been complaining of severe muscular weakness and moderate stiffness and that varied during the day; at first, these diminished soon after each dose of cortisone was administered. As time elapsed, however, the cortisone was less effective in relieving these symptoms. This pattern of events suggested that a state of hypocortisonism had developed. There were no other side effects of hormonal therapy.

Nodule from Lateral Border of Finger (Q98).—On Feb. 14, 1952, examination showed that the specimen was an elliptical piece of skin, 5 mm. long, 1 mm. wide, and 2 to 3 mm. thick. Serial sections disclosed the presence of a minute nodule, no more than 2 mm. in its greatest dimension. This lesion was located deep in the dermis and subcutaneous tissue (Fig. 3) and incorporated a few acini of a sweat

gland. It was characterized by proliferation of mononuclear cells and fibroblasts, proliferation of vessels, and infiltration with small numbers of inflammatory cells. The mononuclear cells were generally distributed about and closely associated with the newly formed capillaries. The latter stemmed from a minute artery, approximately 40 μ in external diameter. The mononuclear cells were ovoid, had poorly

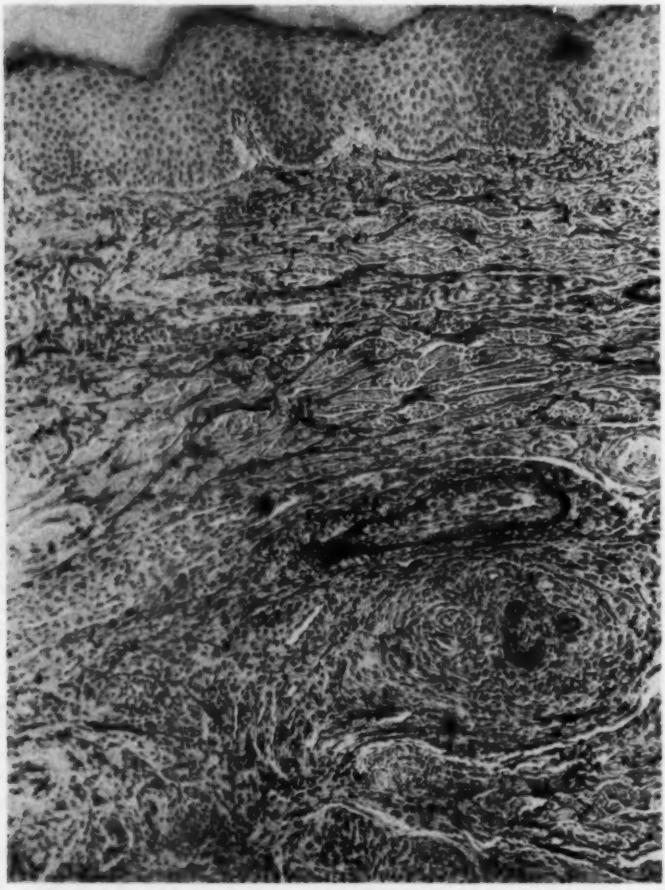

Fig. 3 (Case 2).—Minute nodule, two weeks old, from lateral margin of nail-bed of finger, biopsy number Q98. The nodule of proliferating vessels and cells stems from a minute artery. Its intima is thickened by fibrous tissue that is infiltrated with mononuclear cells and lymphocytes. The remainder of its wall merges imperceptibly with the other elements of the nodule. Weigert's elastic tissue counterstained with hematoxylin and eosin; × 195.

defined borders, and were spaced close to each other. Necrosis was not present. The leucocytes were principally polymorphonuclear neutrophiles and eosinophiles. The intima of the artery was thickened by fibrous tissue. The intima, as well as other layers of the wall, was infiltrated with numbers of lymphocytes and mononuclear cells.

Nodule from Side of Finger (Q113).—On May 14, 1952, examination showed that these sections resembled the preceding ones, except that neither skin nor preformed vessels could be identified. The mononuclear cells were somewhat more pleomorphic than in the preceding sections, and their arrangement was not so compact. Wilder's stain brought out the presence of delicate argyrophilic fibers between the cells.

Nodule from Elbow (Q127).—On June 25, 1952, four blocks of fibroadipose tissue were received. The greatest dimension of any of these was 2 cm. Serial sections revealed confluent lesions that varied in character from area to area. At some points there were well-developed rheumatoid nodules of classical appearance (Fig. 4*A*). Adjacent to these were multiple lobulated areas of vascular proliferation quite similar to those described in the preceding case (Fig. 4*B*). Inflammatory changes and necrosis were seen in many minute vessels (Fig. 5). In the earliest state of this angiitis, mononuclear cells and fibroblasts infiltrated their walls; focal areas of coagulative and hemorrhagic necrosis appeared in their internal aspects. As the process progressed, the landmarks of the wall and lumen were obliterated by the necrosis. Phosphotungstic acid-hematoxylin and Gram stains revealed strands of fibrin to impregnate the wall. The necrosis then extended to surrounding fibrous tissue. This, too, was infiltrated with fibrin and was smudged in places with amorphous basophilic material that presumably was derived from nucleoprotein. Areas of cellular proliferation were present that closely resembled those seen in the nodules obtained from the fingers.

In another section of subcutaneous tissue there was virtually no frank involvement with nodule formation. Many of the lobules of adipose tissue were encroached upon and partially replaced by loose fibrous tissue. In places this was edematous. Clusters of small blood vessels were present in several lobules. Many of these vessels were large capillaries; they had relatively thick walls and were somewhat tortuous. Small numbers of mononuclear and plasma cells were distributed at the periphery of some of these vessels. Several arterioles and small arteries also were present. In one artery that had an external diameter of 48 μ, the intima was extensively vascularized. It was thickened by fibrous tissue and contained four capillary channels. The caliber of the lumen was markedly diminished as a result. The internal elastic lamella was fragmented. The media was intact; the adventitia was slightly thickened by a layer of compact fibrous tissue.

CASE 3.—I. T. was a white man, 36 years old, whose arthritis had begun simultaneously with an attack of acute pericarditis two years previously. Subsequently, joint pains and subcutaneous nodules appeared. Two months prior to the present time, he had been admitted to another institution with fever and limitation of motion of elbows, fingers, and knees. Subcutaneous nodules appeared on the ulnar aspects of the arms. One of these was excised and reported as a "non-specific nodule of the rheumatoid arthritis type." Laboratory tests were normal except for a 3 + cephalin flocculation test and a 2 + thymol turbidity test. No objective changes were noted on physical examination, and roentgenograms of hands, knees, and spine were normal. The erythrocyte sedimentation rate was 32 mm. per hour (Wintrobe). An electrocardiogram was normal, and there were no cardiac abnormalities. The agglutination test for hemolytic streptococci was doubtful. Another subcutaneous nodule was removed. Neither gold nor hormonal medication had been administered. Biopsy of the gastrocnemius muscle revealed the presence of a subacute arteritis. This has been reported previously [6] (Case 5).

6. Sokoloff, L.; Wilens, S. L., and Bunim, J. J.: Arteritis of Striated Muscle in Rheumatoid Arthritis, Am. J. Path. **27**:157-173, 1951.

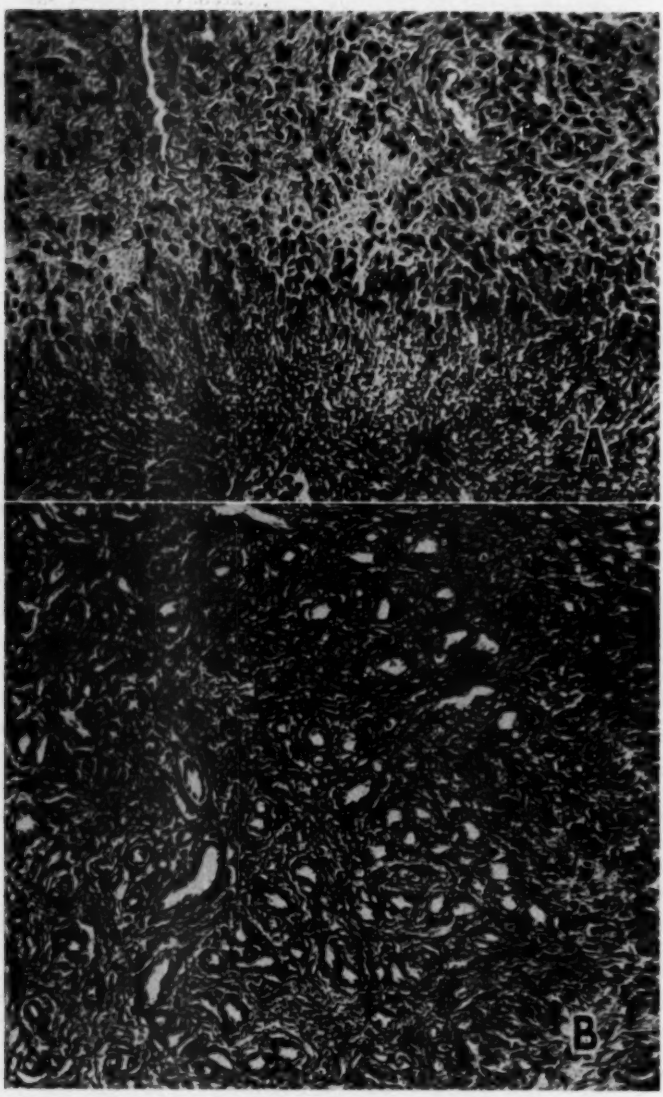

Fig. 4 (Case 2).—*A*, the more mature nodule from the region of the elbow, biopsy number Q127. The zone of necrosis is at the bottom of the photograph; above this is the palisade of radially arranged elongated cells. Hematoxylin and eosin; × 203. *B*, Wilder's preparation brings out the extent of the vascular proliferation in the periphery of the nodule in Figure 4 *A*; × 172.

Subcutaneous Nodule (M182).—The specimen consisted of skin and subcutaneous tissue. It measured 1.6 by 1.1 by 0.5 cm. in greatest dimensions. Histologic sections revealed four distinct nodular lesions in the subcutaneous tissue (Fig. 6). The greatest length of any of these was 3 mm. They were characterized by proliferation of small blood vessels, cellular reaction, and focal necrosis. The vessels were

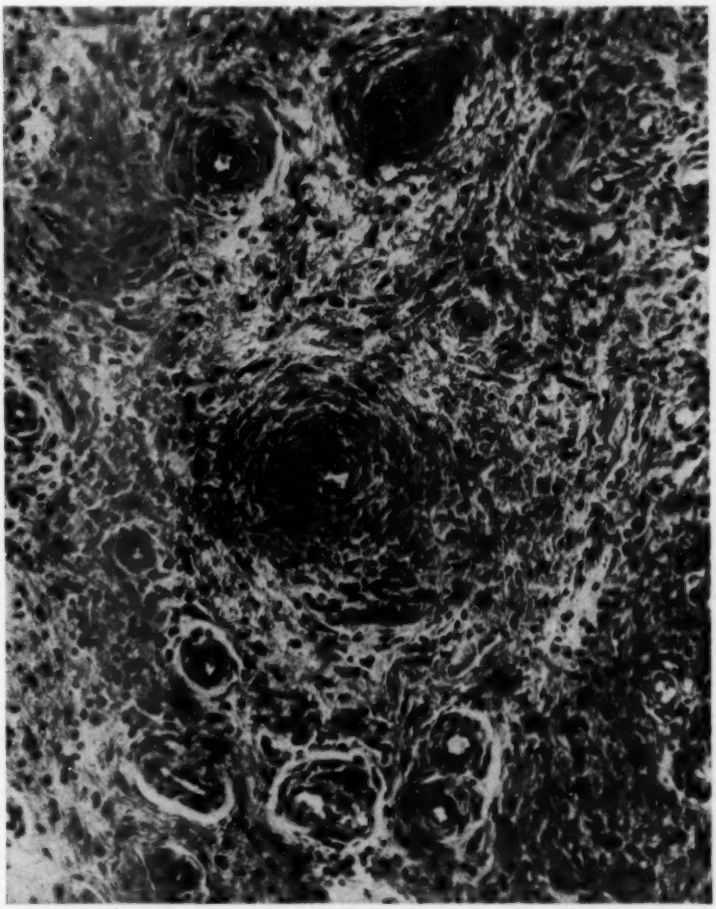

Fig. 5 (Case 2).—Necrosis of vessels in the peripheral, proliferating region. Biopsy number Q127; × 150.

most clearly seen in the central portions of the nodules and constituted approximately one-third to one-half of the substance of the nodules. In some instances, the vessels were small but thick walled; but in most, they were newly formed capillaries. Occasionally an arteriole could be seen penetrating axially into one pole of the nodule of granulation tissue. The cells and the necrosis were most marked in the peripheral portions of these nodules. A large proportion of the cells had undergone

coagulative necrosis, and their precise type was uncertain. Many, however, were obviously polymorphonuclear leucocytes; others, moderate-sized mononuclear cells or young fibroblasts. Occasional eosinophiles were present. In these regions of necrosis, the interstitial connective tissue fibers were swollen, refractive, and eosinophilic. Here and there were smudges of basophilic material of nuclear origin. Only

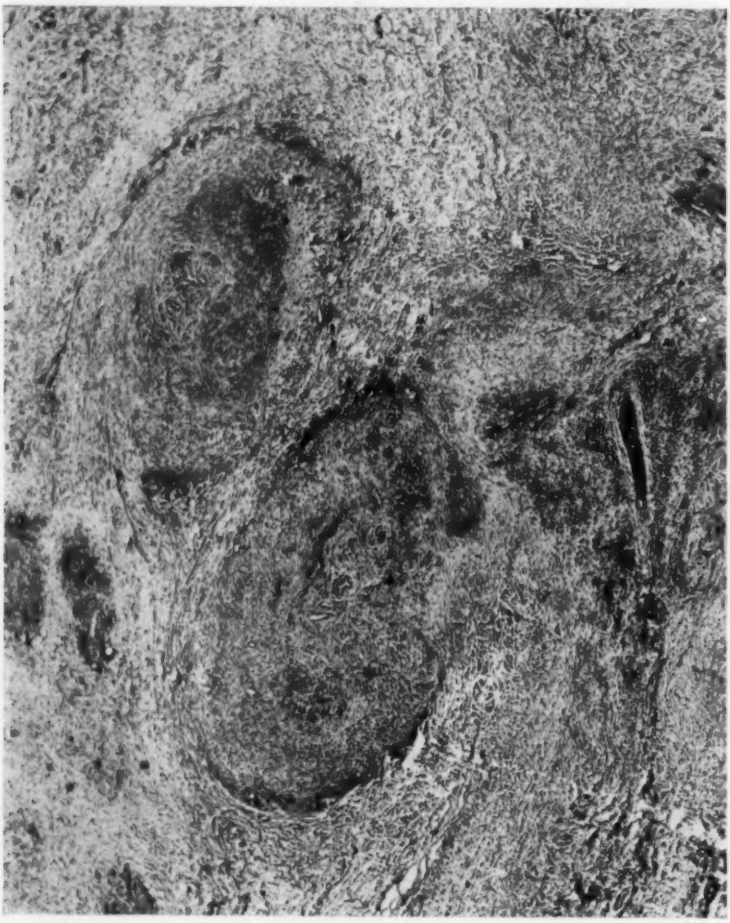

Fig. 6 (Case 3).—The nodules are multicentric. They are distributed about small blood vessels. At the right is a minute vessel that is occluded by a thrombus of hyaline material with a few leucocytes; × 50.

in isolated areas were there well-developed palisades of elongated mononuclear cells. These were radially arranged at the periphery of the nodules of granulation tissue (Fig. 7). The walls of many capillaries were undergoing necrosis. In occasional instances, hyaline thrombus material was present within their lumina as well. Aside from these nodules, minor changes were seen elsewhere in the subcutaneous tissue.

Principally these were characterized by nodular proliferation of capillaries, some edema, and mild fibrosis. Small aggregates of chronic inflammatory cells were clustered about some small vessels.

CASE 4.—M. C., a 30-year-old Negro woman, developed migrating polyarthritis on May 8, 1952. This proceeded to involve the knees, ankles, hands, wrists, elbows, and shoulders. The joint symptoms persisted, and the patient was admitted to the hospital on May 29. At that

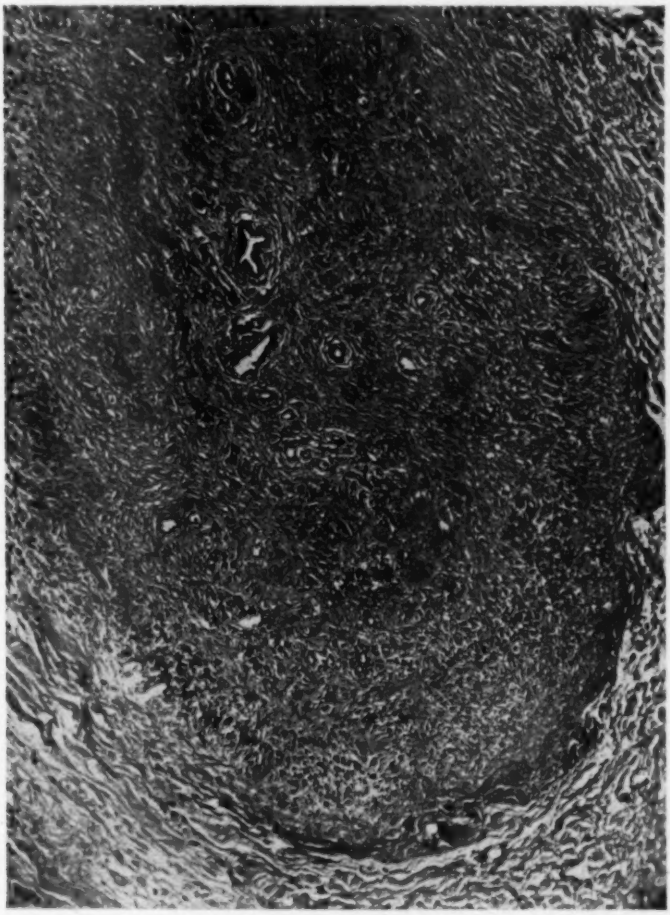

Fig. 7 (Case 3).—A higher magnification (× 144) of one of the nodules in Figure 6. The palisade of mononuclear cells and the zone of necrosis are located at the periphery of the granulation tissue. Many capillaries have necrotic walls. Hematoxylin and eosin.

time effusion was noted in both knees; these were tender, warm, and swollen. The ankles also were warm, swollen, and tender. The proximal interphalangeal joints of the hands and the joints of the wrists, elbows, and shoulders were painful and tender but not warm or swollen. The heart was enlarged; the rhythm regular, 100 per minute. Apical systolic, diastolic, and aortic diastolic murmurs were heard. The electrocardiogram was normal. The patient had had a previous bout of polyarthritis at the age of 12; subsequently she was told by physicians that

she had heart disease. There were no recurrences of arthritis intervening between that and the present illness. On April 10, 1952, four weeks prior to the onset of the present illness, she had had a sore throat that was treated only with saline gargles. The erythrocyte sedimentation rate (Westergren) was 111 mm. per hour. The white blood count was 10,500 per cubic millimeter; 40% polymorphonuclear neutrophiles, 57% lymphocytes, and 3% monocytes. The serum agglutination tests for hemolytic streptococci and for sensitized sheep erythrocytes were negative on June 2, 1952. The antistreptolysin 0 titer was 375 units per cubic centimeter (normal maximum, 150). There was a moderate improvement of joint pain and stiffness in response to a three-day-course of butazoïidine and, after an interval of 11 days, only equivocal relief from acetylsalicylic acid given for 11 days. Over this period of 25 days, the effusion into both knees persisted; the elbows, wrists, and shoulders remained stiff and painful. A subcutaneous nodule, approximately 1 cm. in diameter, appeared on the left ulnar border, 2 in. (5 cm.) distal to the olecranon process, on June 10. It was excised for biopsy on June 25. No other nodules appeared.

Subcutaneous Nodule (Q126).—The specimen grossly measured 6 by 3 by 3 mm. Approximately two-thirds of it were involved in nodule formation. This was characterized by proliferation of undifferentiated, pleomorphic, mononuclear cells and of small blood vessels, on one hand, and by necrosis and a minor inflammatory reaction, on the other. The mononuclear cells were of the variety commonly seen in rheumatic and rheumatoid nodules. Blood vessels constituted approximately one-third of the lesion. Most of these were large capillaries, but several preformed arterioles also existed within the nodule. The walls of these arterioles had a thickened intima because of some swelling and proliferation of endothelial cells. In addition, there was an infiltration of numbers of mononuclear cells into the media and adventitia. Several eosinophilic leucocytes also were present. Gram-Weigert and phosphotungstic acid-hematoxylin stains revealed strands of fibrin to encircle many of the capillaries. In places the fibrin coursed between and surrounded clusters of the vessels, imparting a seemingly lobulated appearance to the granulation tissue. The material staining like fibrin was frequently closely associated with argyrophilic fibrils that presumably were reticulum. Coagulation necrosis of the tissue was taking place in several foci where fibrin had exuded. In some of these the detritus was smudged with basophilic nuclear material.

Case 5.—D. B. was a 44-year-old white woman whose first episode of arthritis occurred eight years before admission to the hospital. This attack was characterized by tenderness and swelling of the joints of the hands and knees. These were accompanied by slight fever that lasted about three weeks. The symptoms then disappeared without treatment. Following this, the patient was well until three months before admission to the hospital, when pain appeared in the left shoulder. During the next few weeks, the fingers, wrists, elbows, knees, and ankles became involved. Physical examination on admission in April, 1946, revealed swelling and tenderness of the proximal interphalangeal joints, the right wrist, the elbows, the knees, and the ankles. No subcutaneous nodules were noted. There was a soft systolic murmur at the apex, but the heart was otherwise normal. The erythrocyte sedimentation rate (Westergren) was 39 mm. per hour. Roentgenograms revealed slight osteoporosis of the bones of the hands without destructive changes in bone or cartilage.

On June 25, 1946, several subcutaneous nodules were observed on the right forearm, just distal to the elbow. On July 15, 1946, a nodule was excised. It was found to be embedded in the anconeous muscle. No medication other than with acetylsalicylic acid had been administered before biopsy. The patient's further course was characterized by more or less continuous pain in involved joints and by gradually increasing deformities of the hands, elbows, and knees. About eight months later, the fourth cervical vertebra became subluxated on the fifth cervical vertebra. Roentgenograms disclosed narrowing of the intervertebral space. At that time there were destructive changes in the interphalangeal joints. During the few months following biopsy, numerous other subcutaneous nodules developed on the arms and scalp.

Subcutaneous Nodule (2505-46).—The specimen comprised two irregularly shaped pieces of pale tissue of rubbery consistence. Their dimensions were approximately 2.5 by 2.0 by 1.5 and 1.5 by 1.0 by 1.0 cm., respectively. The larger piece of tissue was covered on one aspect by skin.

Sections were made from three blocks of tissue. In each there was a widespread process of nodule formation. With the exception of a relatively small mature area, this was characterized by confluent, nodular proliferation of cellular granulation tissue. Between these nodules there was a moderate degree of edema of fibrous tissue. Here the collagen fibers were spread apart from each other; a small number of pleomorphic, fairly large mononuclear cells and fibroblasts were growing parallel to these fibers. In the granulation tissue, the cells were of similar type but more numerous. In places they were associated with much production of collagen. A scattering of polymorphonuclear eosinophiles and neutrophiles was present. About some of the vessels were moderate numbers of plasma cells and lymphocytes. The granulation tissue in most places was highly vascular. In many areas these capillaries had become necrotic, and their walls were converted, to a variable degree, into masses of "fibrinoid" material. In some instances this material occluded their lumina. In others, thrombi were present; these thrombi were largely hyaline but also contained a few erythrocytes and leucocytes. Some of the necrotic vessels were of greater dimension and were thick-walled; presumably these were arterioles. A conspicuous feature of many of these nodules was the occurrence of a well-defined zone of necrosis at their periphery (Fig. 8*B*). With phosphotungstic acid-hematoxylin and Gram stains, material staining like fibrin was found in this zone. In some of the nodules of granulation tissue there was a palisade of radially arranged cells as well. In the area of more mature nodule formation, referred to previously, the zones of necrosis, palisade formation, and fibrosis were more sharply delimited. They coalesced with each other so that it was not always possible to ascertain whether the necrosis occupied a predominantly central or peripheral position.

Case 6.—F. D., a 44-year-old white man, had polyarthritis since January, 1951, three months before coming under our observation. He had lost 10 lb. (4.6 kg.). His hands and feet were cool and moist with excessive perspiration. The ankles and the right knee were warm, swollen, and tender. There was an effusion into the right knee; the synovial fluid was somewhat viscous, yellow, and turbid. It contained 6,400 leucocytes per cubic millimeter, of which 44% were polymorphonuclear neutrophiles and 56% lymphocytes. Roentgenograms disclosed demineralization of the juxta-articular bone and destruction of the articular cartilages of the knee and the tarsal joints of the feet. The serum agglutination test for hemolytic streptococci was positive. The test for sensitized sheep erythrocytes was negative. The erythrocyte sedimentation rate (Westergren) was 52 mm. per hour. On March 21 an early subcutaneous nodule was observed over the right ulna near the olecranon process. Biopsy was performed on March 29. No antirheumatic therapy had been instituted.

Subcutaneous Nodule (Q82).—The specimen was an ovoid mass of soft tissue measuring 2.0 by 1.0 by 0.4 cm. Sectioning revealed most of it to be loose fibrous tissue surrounding some lobules of adipose tissue. In one of two blocks, most of the connective tissue was enormously distended with proteinaceous edema fluid. Only at one point of the periphery of the zone of edema were a few elongated mononuclear cells lined up in radial fashion. In a small number of serial sections, a lesion was found that had the usual appearance of a well-developed rheumatoid nodule. Its greatest dimension was 6 mm.; two central zones of necrosis, 1 to 2 mm. in width,

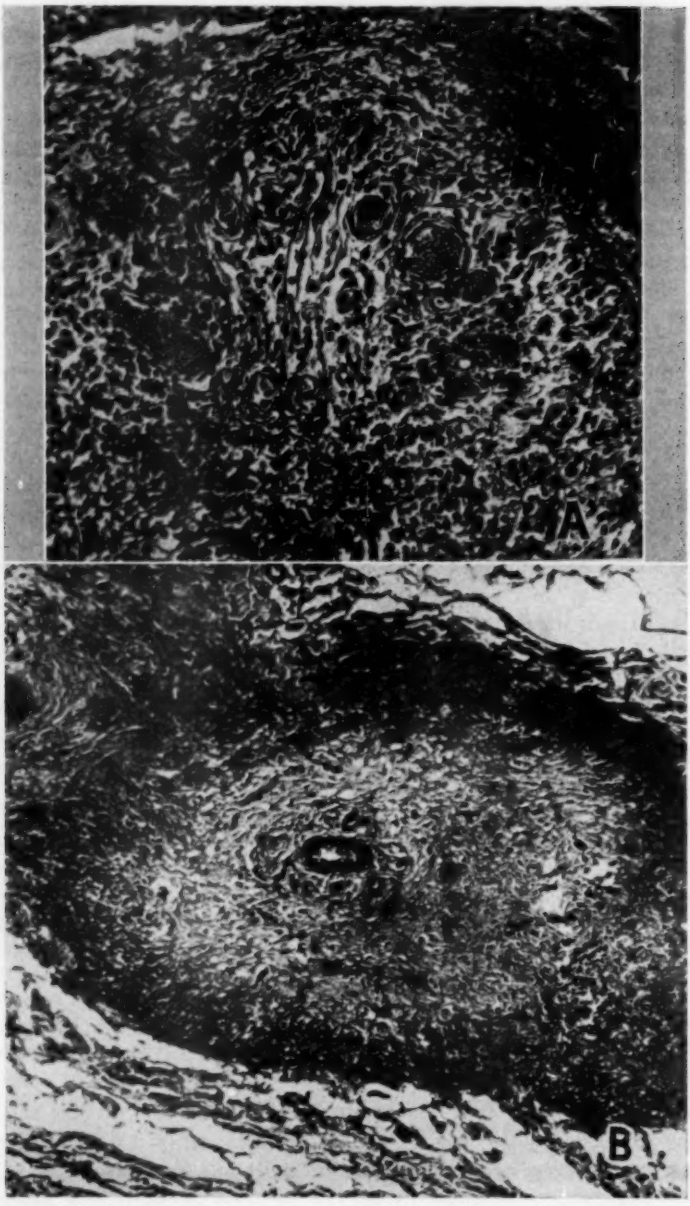

Fig. 8.—*A*, Case 4, a characteristically vascular portion of the young subcutaneous nodule. In the upper right corner necrosis is taking place in the peripheral portion of the granulation tissue. Hematoxylin and eosin; × 182. *B*, Case 5, a nodule of granulation tissue surrounds a central arteriole. The walls of the arteriole and many capillaries are undergoing necrosis. The darkly staining periphery of the nodule is a zone of necrosis. The adjacent fibroadipose tissue, in large part, retains its affinity for the fuchsin of Van Gieson's stain; × 115.

were present within it. The peripheral fibrous zone, although relatively narrow, was quite vascular. In the fibrous tissue, at some slight distance from this nodule, were numerous lobulated clusters of capillaries. These were separated from each other by small amounts of loose fibrous tissue, a few mononuclear cells, fibroblasts, and an infrequent polymorphonuclear neutrophil. In some instances these capillaries were confined to lobules of adipose tissue; elsewhere they coursed between bundles of compact collagen.

The following case is included, not because the nodules were in an early state of development, but because vascular lesions were present within the nodule as well as striated muscle and synovial membrane.

CASE 7.—C. S. was a white man aged 59 whose arthritis began five years prior to admission to the hospital. At the onset, the feet, ankles, and knees became warm, red, and swollen. A spontaneous remission occurred nine months later. After several months the arthritis recurred, extended to many peripheral joints, and persisted until the present admission. At the time of the recurrence, 1948, several subcutaneous nodules appeared near the elbow. These persisted until the time of biopsy, Feb. 6, 1952. Gold salts were administered in 1948 for several months but exerted no effect. In the past five years, the patient lost 13 lb. (6 kg.). On examination in January, 1952, there was an effusion into the knees and the subdeltoid bursae. The proximal interphalangeal and metacarpophalangeal joints of the hands and the joints of the wrists and elbows were swollen and stiff. Several large subcutaneous nodules were present over the free border of each ulna. The serum agglutination tests for hemolytic streptococci and sensitized sheep erythrocytes were positive in February and March, 1952. On Feb. 6, 50 mg. of hydrocortisone was injected into the left subdeltoid bursa. No other medication was administered until after Feb. 25, when biopsy of the left calf muscle was performed. Synovial membrane was obtained by needle punch on Feb. 12.

Subcutaneous Nodule (Q91).—Serial sections revealed the lesion to be a well-developed subcutaneous nodule of rheumatoid arthritis. It was approximately 2 cm. in diameter. In the peripheral zone of fibrosis, at some distance from the zone of necrosis, a small artery (approximately 40 μ in external diameter) was involved in an inflammatory process. Large numbers of mononuclear cells infiltrated all the layers of the wall, but principally the adventitia and perivascular fibrous tissue were infiltrated (Fig. 9*A*). An area of necrosis was present in the intima. Small numbers of fragmented leucocytes and fibrin were seen here. As a result, the lumen was somewhat narrowed. Thrombus material was not present in the lumen.

Gastrocnemius Muscle (Q99).—Many of 100 serial sections of striated muscle revealed segments of small arteries to be acutely inflamed. The process was characterized by infiltration of mononuclear cells in the adventitia and outer portion of the media (Fig. 9*B*). A few polymorphonuclear leucocytes and lymphocytes also were present. Where the lesion was most intense, a small fibrinous and hemorrhagic deposit was seen in the intima, subjacent to the endothelial cells. The lumen nowhere contained thrombus material.

Synovial Membrane of Knee (Q97).—Three fragments of villous synovial membrane revealed two discrete pathologic processes. There was a chronic inflammatory reaction in the synovial tufts. Distributed among infiltrating lymphocytes and plasma cells was a considerable number of large mononuclear and multinucleated cells with somewhat basophilic cytoplasm. Fibrinous material infiltrated within the connective tissue near the surface and in places formed an exudate upon it. In addition, several small vessels, arteries, and arterioles, had become necrotic (Fig.

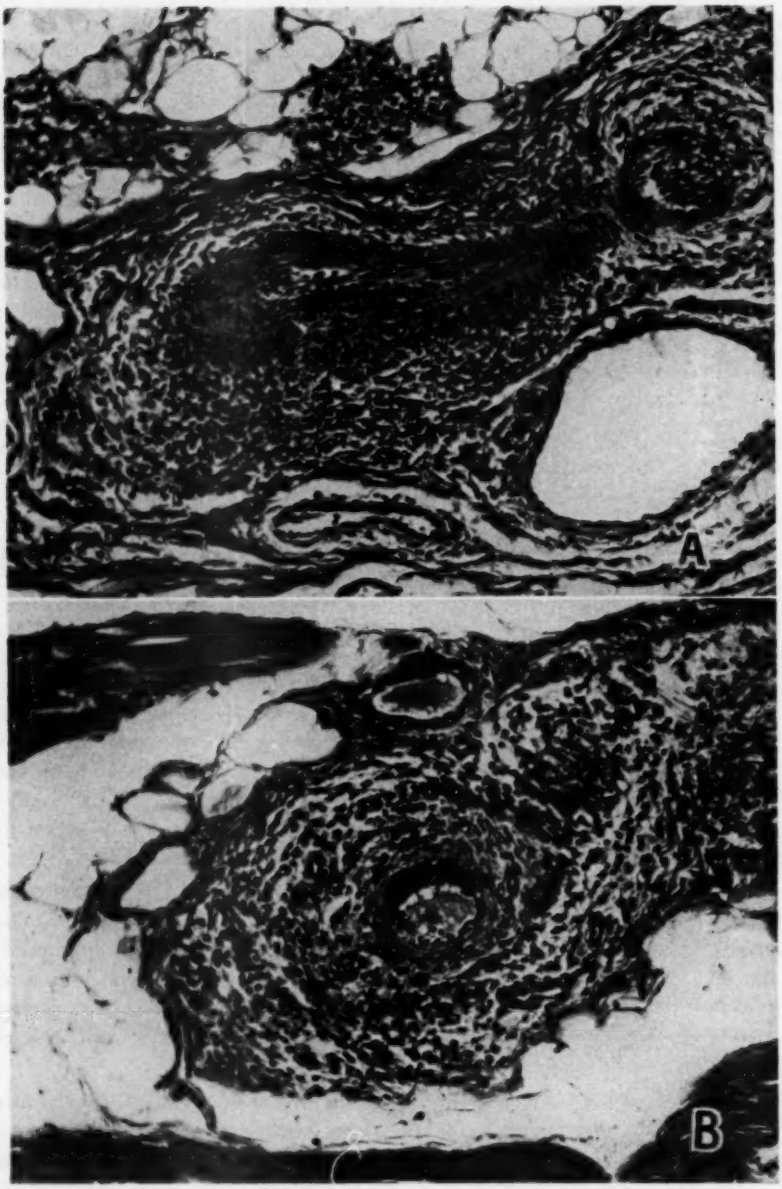

Fig. 9 (Case 6).—*A*, rheumatoid arthritis in adipose tissue adjacent to subcutaneous nodule; × 220. *B*, arteritis of striated muscle; × 320.

10). Gram-Weigert stain revealed their walls to be impregnated with fibrin. The peripheral portions were smudged with basophilic material that probably was derived from nuclear detritus. An infiltrate of inflammatory cells, principally mononuclear and polymorphonuclear leucocytes, was present in the outer aspects of the walls. The endothelial cells were swollen. In some of the vessels, a thick compact

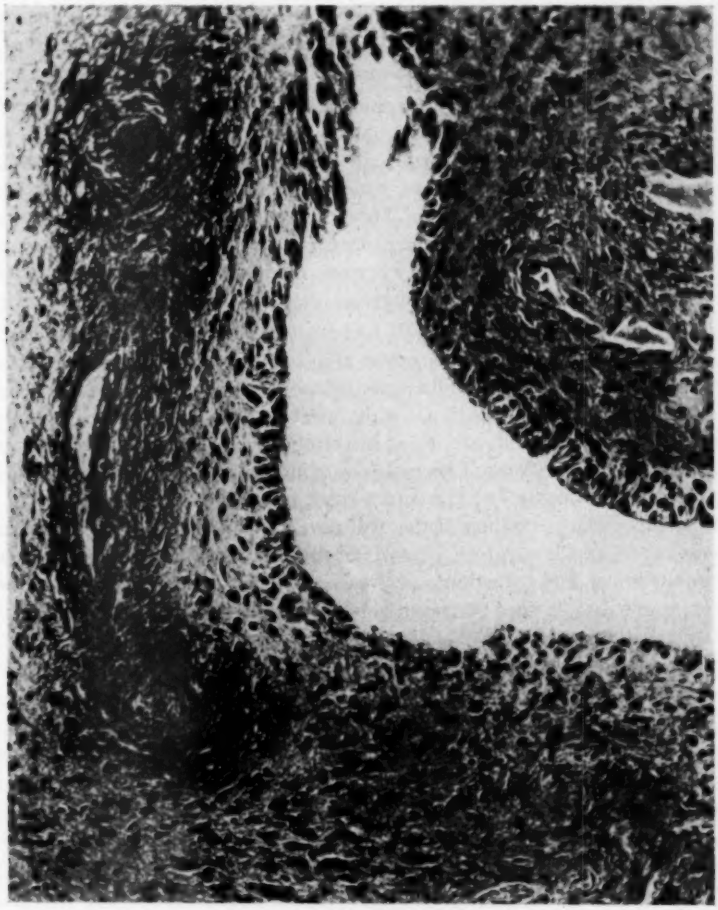

Fig. 10 (Case 6).—Necrosis of arteriole in synovial membrane of knee. The synovitis has a granulomatous character resembling that of the subcutaneous nodule; × 220.

layer of fibrin was adherent to the intimal surface, but the fibrin extended only very slightly into the walls. In other vessels, in addition to the fibrin on the intima, fibrin strands extended through the wall and into the surrounding tissue for a short distance. The lumen of one arteriole was obliterated by fibrin that also infiltrated the wall. In another vessel, the lumen was occluded by a fibrinous thrombus, although the wall was intact.

COMMENT

These cases illustrate four principal features concerning the vessels of the subcutaneous nodule of rheumatoid arthritis.

1. Proliferation of a vascular granulation tissue appears to be an early and integral feature of the development of the nodule. In large part, the newly formed vessels are capillaries that lack any histologic peculiarity. As the lesions become older, as indicated by association with appreciable amounts of collagenous tissue and the cells characteristic of the nodule, the vessels acquire thicker walls of concentrically arranged endothelial cells and fibroblasts. They have no elastic lamella.

2. The characteristic process of necrosis and formation of palisades of radially arranged, elongated mononuclear cells occurs about preformed as well as newly proliferated blood vessels. One ordinarily regards the formation of granulation tissue as a reparative response to destruction of tissue. In the present lesion there is a somewhat unique circumstance: The occurrence of necrosis at the periphery of the vascular tissue suggests that the process is a centrifugal one in relation to the vessels. The fact that the process of necrosis apparently follows the plane of the connective tissue about the vessels suggests that the necrosis-producing agent is fluid-borne into them from the vessels.

3. Inflammatory changes and necrosis may be seen within the vessels in these nodules and in the adjacent subcutaneous tissue. Small arteries and arterioles are involved, as well as the capillaries. In the arteries, inflammatory infiltration predominates; mononuclear cells are found in various layers of the wall but principally in the adventitia. Focal areas of necrosis occur in the intima. These are of a coagulative type; small amounts of fibrin and a small number of red cells exude as well. In two instances vascularization of the wall developed in the wake of such changes. In arterioles and capillaries, total necrosis of the wall may occur; this may at times obliterate the lumen and extend into perivascular tissues. The necrosis of the vessels is a discrete process. It does not result simply from extension of the necrosis of the granulation tissue.

4. In several instances the vascular lesions in the nodules have occurred in individuals in whom it was possible to demonstrate similar lesions in the striated muscle and synovial membrane. The occurrence of arteritis of striated muscle in rheumatoid arthritis has been described previously.[7] The arteritic lesion resembles that described in rheumatic fever [5] more closely than it does polyarteritis nodosa. Thrombosis, aneurysm formation, and infiltration with eosinophiles are not characteristic of rheumatoid arteritis; necrosis of the arterial wall is far less conspicuous. From our personal observations and those of others,[8] it must be admitted that it appears that there may be some predisposition for polyarteritis nodosa to develop in patients with rheumatoid arthritis. While it cannot be denied that the occurrence of polyarteritis nodosa in these individuals may result from

7. Cruickshank, B.: Focal Lesions in Skeletal Muscles and Peripheral Nerves in Rheumatoid Arthritis and Other Conditions, J. Path. & Bact. **64**:21-32, 1952. Footnote 6.

8. Dawson, M. H., in Hench, P. S., and others: The Problem of Rheumatism and Arthritis: Review of American and English Literature for 1938, Ann. Int. Med. **13**:1655, 1940. Graef, I.; Hickey, D. V.; Altmann, V., and Rosenthal, J.: Cardiac Lesions in Rheumatoid Arthritis, Proceedings of the New York Pathological Society, Feb. 26, 1948; Case Records of the Massachusetts General Hospital (Case 37301), New England J. Med. **245**:147-151, 1951.

medication, no constant therapeutic factor can yet be implicated. It would seem that the predisposition is in some manner related to the intrinsic nature of the disease or to the constitution of patients with rheumatoid arthritis. Such persons, also, apparently are somewhat more prone to have rheumatic heart disease, disseminated lupus erythematosus, and chronic peptic ulcer than the general population.

A number of early lesions were included in the extensive study of the subcutaneous nodules of rheumatoid arthritis by Bennett, Zeller, and Bauer.[8] The presence of thrombosed vessels in the younger nodules suggested to these investigators that injury of blood vessels may sometimes be an important factor in the development of the necrosis. It was recognized, too, that remnants of preexisting blood vessels occurred in the necrotic masses of the mature nodule.

On one hand, these observations suggest that the blood vessels play a special role in the pathogenesis of the subcutaneous nodule; on the other, they indicate that the occurrence of vascular lesions in the nodules is a local manifestation of a rheumatoid arteritis that also occurs in the striated muscle and synovial membrane. Inflammatory involvement of systemic arteries in rheumatoid arthritis, other than polyarteritis nodosa, has been reported in a few instances. In our experience this is less frequent, apparently, than in Christie's.[9] The arteritis is regarded as a specific manifestation of the disease. This observation furnishes one more point of similarity to the findings in a group of diseases in which polyarthritis (or arthralgias), disseminated arteritis, and destruction of collagenous tissue are conspicuous. These include rheumatic fever, disseminated lupus erythematosus, serum sickness, and polyarteritis nodosa.

In the early phases of its development, the subcutaneous nodule of rheumatoid arthritis resembles that of rheumatic fever more closely than it does in the mature state. In the nodule of rheumatic fever, there is also a prominent proliferation of capillaries. Indeed, this is an essential histological difference between the subcutaneous lesion and the myocardial Aschoff body of rheumatic fever. The degree of vascularity of the rheumatic and early rheumatoid nodule is quite comparable. Histological differentiation between the two rests principally upon the greater extent of necrosis in the latter.

Although, like other granulomatous lesions and tumors in the subcutaneous tissue, the nodule undoubtedly evokes some secondary formation of new vessels and fibrous tissue, the vascular proliferation in the present instance is of such magnitude and is so closely associated with the development of the nodule that it cannot be regarded as primarily nonspecific. The precise relationship of the formation of new capillaries to that of the cells and production of collagen is not established by these studies. There are good reasons to believe that the cells are young connective tissue cells that presumably become differentiated into fibrocytes. This is suggested by the cytological studies of McEwen[10] and the observations of Kuttner[11] that tissue culture of rheumatic and rheumatoid nodules yields a growth of fibroblasts

9. Christie, G. S.: The General Changes in Rheumatoid Arthritis, in Studies in Pathology Presented to Peter MacCallum, edited by King, E. S. J.; Lowe, T. E., and Cox, L. B., Jubilee, Victoria, Melbourne University Press, 1950.

10. McEwen, C.: Cytologic Studies on Rheumatic Fever: A Comparison of Cells of Subcutaneous Nodules from Patients with Rheumatic Fever, Rheumatoid Arthritis and Syphilis, Arch. Path. **25**:303-314, 1938.

11. Kuttner, A. G.: Personal communication to the authors.

(although this is less luxuriant in the latter than the former). Thus the elements concerned in the proliferative aspects of the nodule are those of banal granulation tissue. It would appear that the development of the nodule involves a stimulus to the proliferation of granulation tissue, as well as a localized disorder in its formation. The latter apparently depends upon the diffusion of a necrosis-producing agent from the blood stream into the perivascular tissue. So far as is known at present, this is not a generalized disturbance in the formation of granulation tissue in patients with rheumatoid arthritis since these individuals respond in no peculiar manner to surgical or other stimuli to the formation of scar tissue. The local factors that are responsible for the formation of the nodule are unknown. Repeated trauma due to pressure would appear to be important.

The principal controversies concerning the pathogenesis of the subcutaneous nodule have centered about the sequence of events in the necrosis and proliferation of connective tissue. That necrosis is the primary event is a view that has been championed by Klinge.[12] Others believe that the necrosis takes place in newly formed collagenous tissue. So far as the present observations disclose, necrosis appears to take place predominantly in the granulation tissue. Although focal necrosis of preexisting bundles of collagen fibers is frequently seen, it usually is of minor extent, and there is nothing in the appearance of the nodules to prove that this has taken place prior to the formation of the nodule. These necrotic bundles of collagen are not found consistently, nor, when they are present, are they always surrounded on all aspects by the granulation tissue. The necrosis may result in part from sequestration of the collagen bundles in the granulation tissue and in part from the action of the lethal agent that also acts on the granulation tissue. This agent does not exert its effect exclusively on the collagenous tissue but also on the blood vessels (including their smooth muscle and endothelial cells), adjacent striated muscle, and adipose tissue.

So far as one can speak of a proliferative phase in the development of the nodule, it involves stimulation to growth of endothelial, as well as fibroblastic, cells. This is, perhaps, not the same as saying that formation of vascular granulation tissue is the primary event in the formation of the nodule or that the factor that causes the necrosis is necessarily the same one that stimulates the proliferation. If there is a morphologic change that precedes the development of the granulation tissue, its character has not been clearly demonstrated here. The only other feature that may conceivably be concerned is the occurrence of localized edema of the subcutaneous tissue in the region of these nodules. This apparently does not have the character of the so-called mucinous edema that is frequently seen in the connective tissues of the heart in rheumatic carditis. Staining with toluidine blue has failed to bring out metachromasia in these regions in the rheumatoid nodule. It is true, however, that special precautions to preserve metachromatic substances were not taken during fixation. Altshuler and Angevine have reported that metachromatic material is associated with the "fibrinoid" of the subcutaneous nodule of rheumatoid arthritis.[13] Exudation is quite characteristic of the subcutaneous nodule of rheu-

12. Klinge, F.: Der Rheumatismus: Pathologisch-anatomische und experimentell-pathologische Tatsachen und ihre Auswertung für das ärztliche Rheumaproblem, Erg. allg. Path. **27**:1-351, 1933.

13. Altshuler, C. H., Angevine, D. M.: Histochemical Studies on the Pathogenesis of Fibrinoid, Am. J. Path. **25**:1061-1077, 1949.

matic fever. The other similarities in the appearance of the young rheumatoid nodule to that of the preceding nodule suggest that the edema in the present instances may be an integral part of the process of development of the nodule. The vascular damage, clearly seen in the angiitic lesions, may provide a background for exudation. The edema may also play a role in the early, nonspecific fibrosis of the subcutaneous tissue. Normally, the latter is primarily adipose. Even in the young subcutaneous nodule, lobules of adipose tissue are seen to be undergoing atrophy with progressive peripheral increase of fibrous tissue. In these regions the fibrous tissue is loose and edematous. At some distance the bundles become more compact but are generally wavy and not hyaline.

That the process of necrosis begins in areas of newly formed granulation tissue accounts for the fact that the nodules are frequently multicentric. Although the necrosis in the early nodule occurs largely at the periphery of the vascular tissue, it occupies a central position in the mature nodule where the foci become confluent and the destruction more extensive.

SUMMARY AND CONCLUSIONS

In the early phases of its development, the subcutaneous nodule of rheumatoid arthritis consists largely of vascular granulation tissue. The characteristic processes of necrosis and formation of palisades of radially disposed elongated cells progress centrifugally about preformed and newly proliferated blood vessels. Inflammatory changes and necrosis may be found in the vessels of these nodules. Similar arteritis may be observed in the striated muscle and synovial membrane in these cases. These findings suggest that the blood vessels play a special role in the pathogenesis of the subcutaneous nodule. Furthermore, they indicate that the occurrence of vascular lesions in the nodules is a local manifestation of a more generalized, specific rheumatoid arteritis.

NOTE:—Since this paper was submitted for publication, a biopsy of the gastrocnemius muscle in Case 2 was performed. In several small arteries there were fibrosis and vascularization of the intima resembling the changes described in the subcutaneous vessels (Q127). Thus, arteritic lesions were present in each of the three instances in which this tissue was examined.

RELATION OF GLYCOGEN, PHOSPHORYLASE, AND GROUND SUBSTANCE TO CALCIFICATION OF BONE

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THREE years ago, Gutman and Yü [1] summarized the evidence which indicated that there was a local enzymatic factor involved in the deposition of calcium in bone. The enzymes were members of the glycogenolytic cycle. Their action was somehow to increase the concentration of phosphate ions to the point where the solubility product of calcium phosphate would be exceeded and precipitation of the salt take place. The source of the phosphate was believed to be a phosphate ester derived from glycogen. Glycogen has been recognized in cartilage cells for a long time, and the literature has been thoroughly summarized by Harris.[2] Follis [3] has shown more recently that rachitic cartilage is deficient in glycogen except in the zone of the most recently hypertrophied cells, where calcium is deposited during the healing of the rickets. As for the enzymes, alkaline phosphatase has been identified in sites of calcification chemically and histochemically (Robison,[4] Gomori,[5] and Gutman and Yü [1]). In addition, the occurrence of phosphorylase in such sites has been demonstrated by indirect methods (Gutman and Gutman [6]). In this paper, evidence is presented to show histochemically the presence of phosphorylase activity in sites of calcification.

The work of Cori and Cori [7] on the enzymatic breakdown of glycogen has suggested that in the presence of phosphorylase under certain conditions the following equilibrium exists:

$$\text{Glycogen} + \text{phosphate ions} \underset{\text{phosphorylase}}{\rightleftharpoons} \text{glucose-1-phosphate}$$

If the reaction is driven to the left (by reducing the phosphate ion concentration), glycogen will form. When this reaction is performed on a section of bone

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1. Gutman, A. B., and Yü, T. F.: Conference on Metabolic Interrelationships, Transactions of the Second Conference, 1950, New York, Josiah Macy, Jr., Foundation, 1950, p. 167.

2. Harris, H. A.: Nature **130**:996, 1932.

3. Follis, R. H., Jr.: Conference on Metabolic Interrelationships, Transactions of the First Conference, 1949, New York, Josiah Macy, Jr., Foundation, 1949, p. 27.

4. Robison, R.: Biochem. J. **17**:286, 1923.

5. Gomori, G.: Am. J. Path. **19**:197, 1943.

6. Gutman, E. B., and Gutman, A. B.: Proc. Soc. Exper. Biol. & Med. **48**:687, 1941.

7. Cori, C. F., and Cori, G. T.: Ann. Rev. Biochem. **10**:151, 1941.

and the section is subsequently stained for glycogen, an increased amount of this substance appears in certain sites as compared with appropriate controls. It is presumably at these sites that the relatively immobile phosphorylase has synthesized the similarly insoluble polymer, glycogen.

There are several reasons for the belief that, while enzymatic activity furnishes a mechanism favoring the precipitation of calcium phosphate, other factors operating locally must be invoked. Phosphatase, for example, occurs intracellularly, as well as extracellularly, in the complete absence of calcification. Phosphatase, demonstrated histochemically, does not reach a peak of activity coincidentally with the calcification of cartilage.[8] Renal calcification takes place at a time when phosphatase activity is greatly reduced or absent.[9] Phosphatase activity is most prominent in certain sites when calcium carbonate, and not phosphate, is being deposited.[10] Thus, the occurrence of phosphatase and of calcification are to a considerable extent dissociable events, and one is encouraged to look elsewhere for a direct local factor in calcification. It would seem that the matrix might possess some special affinity for the calcium salts. This is borne out by Waldman's [11] finding that the matrix of the bone and hypertrophied cartilage will calcify even after heating the tissues to 65 C. or exposing them to enzyme poisons, provided that the calcium and phosphate levels of the surrounding fluids are kept high enough.

According to Gersh and Catchpole,[12] ground substances throughout the body may be conceived as essentially highly polymerized substances containing polysaccharide-protein units that may vary in their degree of aggregation in different regions of the body. Since the carbohydrate groups of the glycoprotein molecules are responsible for the color reaction in the Hotchkiss method, the depth of the stain may be used, under certain conditions, for inferring the degree of polymerization and the amount of packing of the chains or polymer networks in the matrix. It is believed that a deeper stain arises when partial depolymerization allows more reactive groups to become available. In this study, changes were noted in the staining of the matrix of the bone and cartilage which are believed to reflect altered states of polymerization and to be related to the process of calcification. In this connection, it may be noted that, by a kind of competition, calcification of bone has been prevented by combining the bone matrix with toluidine blue.[13]

It seems possible that glycogen, a portion at least of the glycogenolytic enzymes, and the nature and state of the ground substance of bone are intricately involved in the process of calcification. The hypothesis has recently been proposed that phosphatase may be intimately related to the formation of mucopolysaccharides.[14] One might suppose, then, that the enzymes phosphatase and phosphorylase exert their chief effects in calcification through the mobilization and orientation of the ground substance of bone.

8. Siffert, R. S.: J. Exper. Med. **93**:415, 1951.

9. Gersh, I., and Johnson, F. B.: Personal communication to the author.

10. Bevelander, G.: Conference on Metabolic Interrelationships, Transactions of the Third Conference, 1951, New York, Josiah Macy, Jr., Foundation, 1951, p. 222. Romanoff, A. L.: The Avian Egg, New York, John Wiley & Sons, Inc., 1949.

11. Waldman, J.: Proc. Soc. Exper. Biol. & Med. **69**:262, 1948.

12. Gersh, I., and Catchpole, H. R.: Am. J. Anat. **85**:457, 1949.

13. Miller, Z. B.; Waldman, J., and McLean, F. C.: J. Exper. Med. **95**:497, 1952.

14. Moog, F., and Wenger, E. L.: Am. J. Anat. **90**:339, 1952.

A study of this hypothesis was undertaken by a description of the distribution of phosphorylase and of the state of the ground substance. The distribution of phosphotase in the long bones with Gomori's method [5] confirmed that described in the literature. The studies were made on the tibia of normally developing young rats and on the same bone of rats on a rachitogenic diet. Some animals of the latter group were treated so as to induce bone healing.

MATERIALS AND METHODS

The tibias of rats aged from newborn to 45 days were used. Normal rats were fed an adequate stock diet. Rachitic rats of ages between 6 and 8 weeks were obtained.[15] The rickets of some of the rats was partially alleviated by intraperitoneal injections of phosphate ions. The tibias of these rats were also studied.

The rats were killed by a sharp blow on the head and the tibas removed at once. The bones were split lengthwise and both halves dropped into a container of isopentane maintained at a temperature of about — 150 C. The frozen bones were then placed in a vacuum tube housed in a refrigerated chamber at about — 30 C. and allowed to dry *in vacuo* for a period of not less than three days.

The dried bones were dropped into melted carnauba wax (chloroform-soluble fraction) and the tube evacuated. The use of this hard wax permitted the sectioning of undecalcified bones. The wax was kept melted with a low flame during the 15 minutes required for infiltration. The blocks were sectioned at 8μ. The carnauba wax was removed with chloroform prior to staining.

Staining.—The Hotchkiss [16] procedure was used. In this, adjacent hydroxyl or hydroxylamino groups of the tissue carbohydrates are oxidized to aldehydes with periodic acid, and the aldehydes are combined with leucofuchsin to form a bluish-red compound. Various interfering substances, such as glycolipids and simple sugars, may be present, but in this study appropriate controls indicated that glycogen and glycoproteins were the only stainable structures in the sections.

Hydrogen Ion Concentration.—The influence of solutions of various pH values upon bone glycogen and glycoproteins was studied. Buffers were prepared, adjusted to the desired pH in the range from 1 to 11 by 0.5 pH unit steps. Serially cut sections were placed in Petri dishes and each covered with five drops of the appropriate buffer. After standing at room temperature for one hour, each section was rinsed with its original buffer and placed in absolute alcohol overnight before staining.

Phosphorylase.—The following steps were employed to form glycogen at the site of phosphorylase activity:

1. Mounted sections were immersed in chloroform for 10 minutes in order to remove the wax and partially to inactivate bone alkaline phosphatase.

2. Sections were incubated with amylase or saliva for 1½ hours at 37 C. to remove glycogen.

3. Sections were soaked in distilled water overnight to dissolve all soluble bone salts, especially phosphates, which might impede the reaction.

4. Tissue phosphoglucomutase was inactivated by heating the sections, covered with a few drops of water, to 60 C. for 15 minutes in a Petri dish containing a wet blotter.

5. The sections were incubated for six hours in an acetate buffer of pH 5.9 in which crystals of glucose-1-phosphate [17] and barium chloride were dissolved. The pH of 5.9 was chosen

15. Rats were obtained through the courtesy of Dr. W. Bloom and Dr. J. Waldman, of the University of Chicago, Department of Anatomy.

16. Hotchkiss, R. D.: Arch. Biochem. **16**:131, 1948.

17. Furnished by Dr. David Greenberg, Department of Biochemistry, University of California.

empirically as the most effective under these conditions. Glucose-1-phosphate in high concentrations was used to cause a shift to the left in the reaction:

$$\text{glycogen} + \text{phosphate ions} \underset{\text{phosphorylase}}{\overset{\longleftarrow}{\longrightarrow}} \text{glucose-1-phosphate}$$

Barium chloride was used to precipitate phosphate ions as they were released. Incubation was at 37 C.

6. After incubation, sections were placed in absolute alcohol overnight to fix the glycogen and then stained by the Hotchkiss procedure. Since all tissue glycogen is removed by amylase, comparison of an amylase-treated section with a similar one that has undergone the steps listed above permitted the localization of phosphorylase activity through the presence of newly formed glycogen.

Collagenase.—A commercial extract (Lederle) of cultures of Clostridium welchii [18] containing collagenase, hyaluronidase, and other enzymes was used. A mounted section was placed over the depression in a well slide which contained collagenase and a drop of preservative and the edge of the well sealed to the slide with petrolatum. The section was incubated at 37 C. for varying periods up to 72 hours and a control run in a buffer at the same pH. After incubation, the section was stained by the Hotchkiss method. A positive reaction in the bone was signalized by a swelling of the whole spicule and by a uniformly intensified staining of the matrix.

RESULTS

Normal Rats.—Untreated Sections: The epiphysis of the tibia of a newborn rat is cartilaginous, while the diaphysis is formed by a layer of cortical bone enclosing irregularly arranged bony spicules and marrow cells. The small cartilage cells in the distal portion of the growing epiphysis contained dense deposits of glycogen in their cytoplasm, but the cartilage cells on the surface of the bone in the region that would become the articular surface contained no appreciable glycogen. In the deeper portion of the epiphysis the cartilage cells were increasingly swollen and tended to be arranged in columns. The extremely hypertrophied cells were devoid of glycogen except for those just bordering on the bony diaphysis, which contained scattered deposits of glycogen. The intensity of staining of the cartilage matrix was increased progressively with the enlargement of the cartilage cells in the columns. The spicules of bone varied in appearance with their distance from the epiphysis. Those closest to the cartilage had a rather deeply stained matrix, and the osteocytes and osteoblasts contained little or no glycogen in their cytoplasm. The spicules located at a greater distance exhibited a paler matrix along with increased deposits of glycogen in the cells. The bar of calcified cartilage was visible in the center of many of the spicules as an intensely staining red line, whether the bony matrix was pale or deeply colored.

A few changes were noted as the rats increased in age up to 45 days. The matrix of the older bones had a decreasing affinity for the stain, and the epiphyseal plate appeared as the bony epiphysis developed. The innermost articular cartilage cells contained large amounts of glycogen, but those on the synovial surface lacked it, while the cartilage cells in the epiphyseal plate contained glycogen only in the extremely enlarged cells at the periphery of the plate.

Effect of Hydrogen Ion Concentration: Sections of newborn rat bone subjected to solutions of varying pH for one hour showed changes only in the range of pH

18. Obtained through the courtesy of Dr. I. S. Danielson, Lederle Laboratories Division, American Cyanamid Co., Pearl River, N. Y.

4.5 to 5.5. The difference consisted in the absence of glycogen in the majority of bone and cartilage cells, probably through the action of phosphorylase. No changes in the matrix materials were noted.

Collagenase: The bone spicules of sections incubated in collagenase solutions for not less than 72 hours showed swelling accompanied by an increased staining of the matrix. The central core of calcified cartilage could not be differentiated from the abnormally red bone matrix. Sections incubated an equal length of time in solutions of the same pH but lacking collagenase showed at most a slight increase in staining of the matrix.

Phosphorylase: Glycogen formed in vitro on the slide by the method described above may be used as an indicator of the occurrence of phosphorylase. Such newly formed glycogen was present in diffuse form in the cytoplasm of osteoblasts and osteocytes of bone spicules, including the most recently formed ones. The cortical cells showed little evidence of enzyme activity. In cartilage, the greatest amounts were in the somewhat flattened cells that had just begun to hypertrophy and to form columns. The small cartilage cells contained a trace of glycogen, as did the very hypertrophied ones.

Rachitic Rats.—Untreated Sections: The thickness of the cartilage epiphyseal plate was greatly increased over normal. The majority of hypertrophied cells were devoid of glycogen, but there were moderate deposits in the most recently enlarged ones. The matrix of the bone in the abundant osteoid stained more intensely than the matrix of healthy bone of the same age. Osteocytes and osteoblasts contained the same amount of glycogen as, or a greater amount than the normal.

Phosphorylase: Glycogen was found to be formed in sections in osteoblasts and osteocytes, where presumably phosphorylase activity is present. In cartilage, most of the cells contained glycogen at the end of the phosphorylase test, with the least amounts produced by the cells farthest from the epiphysis. This indicates the occurrence of phosphorylase in these cells.

Inoculated Rachitic Rats.—Untreated Sections: The cartilage plate at the epiphysis was measurably narrowed again at the expense of the hypertrophied cells. The glycogen-containing cells of the cartilage were also reduced in number. No change in the staining of the matrix of the bone spicules was observed, but the osteocytes and osteoblasts had in general lost much of their glycogen.

Phosphorylase: There was no definite change from the picture seen in untreated rickets when the slides were treated by the phosphorylase method.

COMMENT

The results of these and other investigations indicate that most, if not all, tissues that normally become calcified possess an organic matrix with certain definite characteristics. The studies of Robinson [19] on bone, using the electron microscope, indicate that there may be a morphological association of salt crystals with the homogeneous, nonfibrillar portion of the matrix, which is similar to the ground-substance found elsewhere in the body. This ground-substance was shown by Gersh and Catchpole [13] to contain polysaccharide-protein substances that can be identified by the Hotchkiss method. With use of this procedure, it is possible to show that in

19. Robinson, R. A.: Conference on Metabolic Interrelationships, 1951, p. 271. Transactions of the Third Conference, 1951, New York, Josiah Macy, Jr., Foundation,

bone matrix, as in ground-substance generally, the units of this material vary in the degree to which they are associated. Thus, an intensely stained area reflects the presence of loosely aggregated groups, while a faint stain indicates a high degree of aggregation or polymerization where the concentration of the material is of the same order.

In the material studied here, it is found that in normal bone the occurrence of a highly stainable matrix precedes or is associated with a light deposition of calcium salts, while heavily calcified areas possess a pale or colorless matrix. The pale matrix in small-celled cartilage reddens as the cells begin to hypertrophy, and calcified cartilage stains darkly even when enclosed by bone. This may be interpreted as either a depolymerization of existing units or the production of new, less completely polymerized matrix. The matrix of newly formed bone is pink, but it loses its staining power upon aging. Cortical bone is pale or colorless. In rachitic bone, the matrix surrounding the hypertrophied cartilage cells stains deeply, as does the osteoid. Collagenase-treated bone acquires a dark stain and becomes swollen, phenomena commonly associated with depolymerization. Engel [20] observe that in teeth the newly formed dentin is dark; it becomes pale in the adult, but stains red in the region surrounding an area of caries.

Heller-Steinberg,[21] who combined the Hotchkiss stain with an improved technique for visualizing bone salts, found that in parathyroid-treated rats, bone undergoing resorption stains darkly in the regions around the osteocytes and radiating canaliculi, as well as at the margins of the trabeculae. The same areas show an increased reaction of the bone salts with dilute silver nitrate, indicating that these are set free as the organic materials break up. She also identified small glycoprotein-containing granules in the osteoblasts and osteocytes of regions where matrix substances were being rapidly formed. These granules disappeared during severe rickets and reappeared upon healing. They were also lacking in the disintegrating bone found in parathyroid-treated rats. Similar granules have been observed by Gersh [22] in fibroblasts believed to be engaged in the secretion of ground-substance in other connective tissues.

At present, it is difficult to assign a definite role to the enzymes phosphatase and phosphorylase shown to be active in bone. Gutman and co-workers [1] have established that rachitic cartilage may be made to calcify in vitro through the action of phosphatase and intermediate enzymes, but it does not necessarily follow that these reactions occur in the living animal. Evidence for the production of certain proteins through the action of phosphatase is accumulating. Bradfield,[23] studying the silk glands of spiders and moths, found that the secreting cells contain phosphatase in the nucleolus and cytoplasm bordering the lumen. He presents evidence that in these and some other tissue cells phosphatase is concerned with the production of fibrillar proteins, such as collagen, by assisting in the liberation of a synthesized protein from its complex with nucleic acid. More specifically, Moog and Wenger [15] report a close association of high phosphatase activity with mucopolysaccharides. Fell and

20. Engel, M. B.: J. Am. Dent. A. **40**:284, 1950.

21. Heller-Steinberg, M.: Am. J. Anat. **89**:347, 1951.

22. Gersh, I.: The Harvey Lectures, Series 45, Baltimore, William & Wilkins Company, 1952, p. 211.

23. Bradfield, J. N. G.: Exper. Cell Res., Supp. 1, p. 338, 1949.

Danielli [24] have shown that in healing wounds of rats an intense phosphatase reaction is given by the fibroblasts, intercellular fibers, and capillaries during the stage of collagen fiber formation.

The relation of cell glycogen and phosphorylase to the manufacture of bone constituents is also obscure. In this study, phosphorylase was most readily demonstrated in glycogen-deficient cells that possessed glycogen at a slightly earlier stage in their development. Thus, the cartillage cells of normal bone that have recently enlarged and formed columns contain little or no glycogen in their cytoplasm, while showing marked phosphorylase activity. Likewise, the glycogen-deficient osteoblasts of the primary spongiosa show phosphorylase activity. Many of the cartilage cells of rachitic rats form glycogen in areas which in the normal state would otherwise lack it. The osteoblasts and osteocytes of older bone spicules show glycogen and demonstrate the action of phosphorylase, but the osteocytes of cortical bone contain glycogen in the apparent absence of phosphorylase activity. Bevelander and Johnson [25] observed a somewhat similar picture in developing intramembranous bone. Looking at the spicules of bone developing from mesenchyme, they found that when the matrix is being rapidly deposited the surrounding osteoblasts lose most of their glycogen, regaining it when the spicule becomes mineralized.

These findings consistently point to the conclusion that the consumption of glycogen through the activity of phosphorylase may possibly be associated with the production of bone matrix and rachitic osteoid and with the production or alteration of cartilage matrix prior to calcification.

SUMMARY

The bones of normally growing, rachitic, and healing rachitic rats were prepared by the freezing-drying method of fixation and imbedded in carnauba wax. This permits the study of undecalcified, minimally altered bone. The Hotchkiss method of staining was employed and controls used to insure the identification of glycogen and glycoproteins.

Phosphorylase was localized by exploiting its ability to form glycogen in sections under appropriate conditions. The sites where glycogen is formed in vitro indicate the locations where phosphorylase occurs.

The glycoproteins of the ground substance in normal cartilage and bone are believed to consist of units that are loosely aggregated (relatively less polymerized) in areas containing, or about to receive, an initial deposit of calcium salts. These units are tightly packed or polymerized in small-celled, hyaline cartilage and mature bone. The ground substance of rachitic osteoid contains intensely staining, slightly polymerized glycoproteins.

The bone and cartilage cells most consistently found to show phosphorylase activity are those that contained glycogen at some period in their development but are temporarily lacking in it. These cells occur in areas showing the most rapid formation of calcifiable matrix glycoproteins.

240 S. Chauncey St.

24. Fell, H. B., and Danielli, J. F.: Brit. J. Exper. Path. **24**:196, 1943.

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EFFECT OF HIGH PYRIDOXINE INTAKE IN CHOLESTEROL-FED CHICKS

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THE OBSERVATION by Rinehart and Greenberg [1] that in monkeys on low pyridoxine diets atherosclerosis developed incriminated pyridoxine as possibly playing a role in the causation or prevention of atherosclerosis. As pointed out by other observers,[2] this bears further investigation.

The present experiment was designed to note the possible prophylactic effect of a high pyridoxine intake in experimental atherosclerosis. It is well documented that high cholesterol and apparently normal pyridoxine intake will result in atherosclerosis in certain experimental animals. The question raised was whether or not an adequate intake of pyridoxine is necessary for metabolism of exogenous cholesterol and prevention of hypercholesteremia. In other words, since high cholesterol intake produces atherosclerosis experimentally, would a concomitant rise in the pyridoxine intake tend to prevent the lesions?

PROCEDURE

Twenty-four white Leghorn cockerels, aged five weeks, were chosen as the experimental fowl. The method of producing atherosclerosis as outlined by Dauber and Katz [3] was used with minor variations. All birds received a basic mash which is used routinely by the Poultry Husbandry Department of the Pennsylvania State College.[4] The pyridoxine content has never been determined but is considered entirely adequate for normal growth and development.

The birds were divided into four groups of six each. The first group received the mash only. The second group received mash plus 1% cholesterol. The third group received mash, 1% cholesterol, and 16.0 mg. of pyridoxine per pound of mash. The fourth group received mash, 1%

The pyridoxine was kindly supplied by the Eli Lilly Company.

Aided by a grant from the Centre County Branch of the Pennsylvania Heart Association.

This work was done with the facilities of the Departments of Biochemistry and Animal Husbandry of the Pennsylvania State College.

1. Rinehart, J. F., and Greenberg, L. D.: Pathogenesis of Experimental Arteriosclerosis in Pyridoxine Deficiency, with Notes on Similarities to Human Arteriosclerosis, A. M. A. Arch. Path. **51**:12, 1951.

2. Moses, C., and Peters, J. H.: Nutrition and Arteriosclerosis, Editorial, Pennsylvania M. J. **55**:1020, 1952. Wakerlin, G. E.: Recent Advances in the Pathogenesis and Treatment of Atherosclerosis, Ann. Int. Med. **37**:313, 1952.

3. Dauber, D. V., and Katz, L. N.: Experimental Cholesterol Atheromatosis in an Omniverous Animal, the Chick, Arch. Path. **34**:937, 1942.

4. Starter mash consisted of the following components in parts per 1,000: ground yellow corn 394, wheat bran 50, wheat middlings 100, ground heavy oats 100, alfalfa meal (low fiber, 50,000 A) 50, soybean oil meal (solvent) 150, fish meal (not less than 55% protein) 25, meat scraps (not less than 50% protein) 50, dried whey 30, riboflavin concentrate (8,000γ) 0.2, brewer's yeast 24, ground limestone (high calcium) 10, steamed bone meal 10, iodized salt 2.5, 300 D-1,500 A fish oil 4, and anhydrosis magnesium sulfate 0.2.

cholesterol, and 80 mg. of pyridoxine per pound of mash. The amounts of pyridoxine were chosen arbitrarily.

In an effort to have healthier birds than those in the experiment of Dauber and Katz,[3] only 1% cholesterol was added to the mash. Also, the cholesterol and pyridoxine were added in a dry powdered state by thorough mixing in a commercial mixer. The feed was constantly available to the birds.

Blood specimens were obtained by cardiac puncture for total cholesterol determinations at the beginning of the experiment and after the third week. Total cholesterol was determined by Reinhold and Shiels' modification of the Myers-Wardell method.[5]

At the end of five months all birds were killed, and the thoracic aortas were sectioned for evidences of atherosclerosis. The specimens were fixed in Zenker's solution, paraffin-sectioned, and stained with hematoxylin and eosin.

RESULTS

During the experiment the general appearance of the birds receiving cholesterol and those receiving cholesterol plus pyridoxine was about the same. These changes

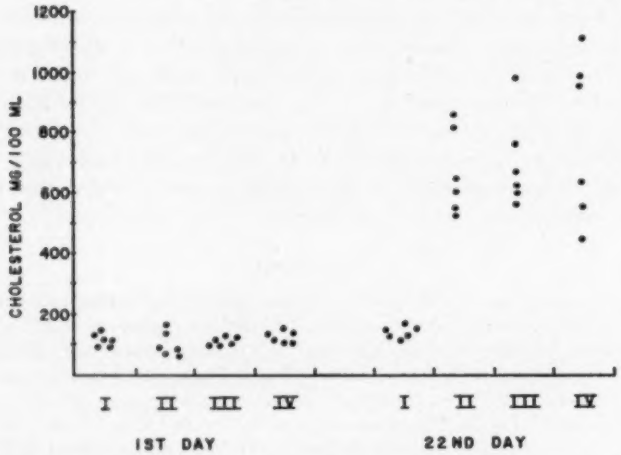

Chart showing the approximate total blood cholesterol values in the four groups on the 1st and 22d days of the experiment.

have been described previously and consisted of yellow-tinged, greasy-appearing, ruffled feathers with underdeveloped musculature. All birds remained moderately healthy and active to the end of the experiment, except one which died a few weeks after the experiment started.

The approximate cholesterol levels are shown in the Chart. At the end of the third week, Groups III and IV, which received pyridoxine, had hypercholesteremia similar to Group II, which received cholesterol without pyridoxine. In other words, the pyridoxine failed to prevent the hypercholesteremia which is such a prominent feature in these experiments.

On sectioning the thoracic aortas at the end of five months, no atherosclerotic lesions were found in the control Group I. Group II, which received cholesterol without pyridoxine, showed moderate numbers of early lesions, as was expected.

5. Hawk, P. B.; Oscar, B. L., and Summerson, W. H.: Practical Physiological Chemistry, Ed. 12, Philadelphia, The Blakiston Company, 1947.

These lesions were not extensive, and the better-developed ones contained only a few layers of foam cells.

However, Groups III and IV, which received pyridoxine, showed numerous well-developed foam cell plaques. These lesions appeared to be more widespread and contained many more layers of foam cells than the plaques in Group II. The intima overlying each plaque was intact, and all showed extension of the fatty material into the adjacent layers of the media. The elastic fibrils directly beneath a plaque were irregularly disrupted. In no case was there evidence of necrosis, ulceration, fibrosis or calcification, indicating the early atherosclerotic lesion so aptly described by Leary.[6]

CONCLUSION

High pyridoxine intake failed to inhibit atherosclerosis in chickens fed cholesterol. In fact, the pyridoxine seemed to enhance the lesions.

Assistance was given by Eugene McKostick in the cholesterol determinations and by Timothy Leary, M.D., Merl G. Colvin, M.D., and Mahlon J. Pophal, M.D., in reviewing the sections.

6. Leary, T.: The Genesis of Atherosclerosis, Arch. Path. **32**:507, 1941.

EFFECTS OF ANTERIOR HYPOPHYSIS ON MAMMARY GLANDS AND ADRENALS

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AND
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IN MICE of strain A orchiectomized at the age of 3 to 4 weeks, homeotransplants of anterior hypophysis stimulated mammary growth. The adrenals of the animals with advanced breast proliferation were not hyperplastic. Therefore, secretions given off by the grafted hypophysis were thought to promote mammary growth.[1] In order to study further the role of the hypophysis and the adrenals in inducing proliferation of the breast tissue, the following experiments were carried out.

MATERIALS AND METHODS

One hundred twenty male mice of the closely inbred strain A raised in our laboratory were orchiectomized at 1 to 3 days of age and, after weaning, were divided into three groups of 40 animals each. First Series: The mice received no further treatment. Second Series: The mice received subcutaneous transplants of four adrenals each, according to a method described previously[2] (8 mice received male adrenals, 27 received female adrenals, and 5 received both male and female adrenals). Third Series: The mice received subcutaneous transplants of four adrenals and four anterior hypophyses (10 mice received adrenals taken from males, 28 from females, and 2 from both male and female donors; 7 mice received hypophyses from male, 25 from female, and 8 from both male and female donors). All mice were kept on a stock diet of Purina Laboratory Chow and water ad libitum.

Two animals of each series were killed one, two, four, and six months after transplantation. Later the mice were killed as their condition warranted it, the oldest ones living to the age of 23 months. At autopsy, internal organs, salivary glands, thyroids, adrenals, legs, mammary glands, and recovered grafts were fixed in Bouin's solution and embedded in paraffin; semiserial sections were made and stained with hematoxylin and eosin. Some hypophyses and hypophyseal grafts were fixed in Regaud's solution for cytologic studies. A few animals that were found dead and whose tissues were autolyzed were excluded from this investigation, and only those mice were studied in which the four mammary glands and adrenals were available for microscopic examination. Details may be seen in Tables 1, 2, and 3.

From the Snodgras Laboratory, Hospital Division, City of Saint Louis.

This investigation was aided by the Brant Fund of the American Medical Association.

1. Silberberg, M., and Silberberg, R.: Mammary Growth in Orchidectomized Mice Grafted with Anterior Lobes of Hypophysis and Ovaries at Various Ages, Arch. Path. **49**:733, 1950.

2. (*a*) Silberberg, M.; Silberberg, R., and Opdyke, M.: Adrenals and Anterior Hypophysis in Leukemogenesis of Mice, Proc. Soc. Exper. Biol. & Med. **82**:10, 1953. (*b*) Silberberg and Silberberg.[1]

OBSERVATIONS

Fate of Transplants.—The adrenal transplants "took" readily. In all six mice examined up to four months after transplantation, the adrenal tissue showed regular architecture. The grafts were surrounded by a thin vascular connective tissue and contained a few foci of mononuclear leucocytes. The longer the grafts were allowed to remain in the recipient, the denser became the connective tissue capsule, and the more pronounced was the lymphocytic infiltration. In some animals, the adrenocortical cells underwent regressive changes, necrosis, and calcification and were ultimately resorbed or replaced by a fibrous knob with a foreign body reaction around cholesterol clefts. The latter apparently resulted from the liberation of lipoid material from degenerating cortical cells. In most animals, however, the grafted adrenals retained their original structure. At later stages hyperplasia of the adrenocortical cells with the formation of small nodules was observed (Fig. 1). These changes resembled those seen in the animals' own adrenals.

Of a total of 35 mice, adrenal tissue was recovered in 27 (77.1%). In animals sacrificed at the age of 7 months and later, the mean age at which grafted tissue was recovered was 15.3 months, with a range from 7 to 23 months. In five grafts cortical nodules were noted; these grafts had survived for periods of 9 to 19 months, with a mean survival of 14.9 months.

In the animals receiving both adrenal and hypophyseal transplants the grafts likewise "took" satisfactorily. This again was demonstrated by the presence of preserved adrenal and hypophyseal tissue in all six animals killed up to the age of 5 months.

Of a total of 37 mice, adrenal tissue was found in 26 mice (70.3%) and hypophyseal tissue in 24 mice (64.9%). The actual number of mice showing hypophyseal grafts was probably even higher since pieces of tissue most suggestive of "takes" were saved for cytologic studies. Disregarding the animals killed up to the age of 5 months, the mean age at which grafted adrenal or hypophyseal tissue was identified was 14.5 months, with a range from 7 to 21 months. In some animals either only adrenal or only hypophyseal grafts were recovered. In 20 mice (54.1%) of the total of 37, both types of grafts were found in the same animal. The mean age of these animals was 13.4 months, with a range from 7 to 21 months. As early as one or two months after transplantation, the adrenocortical cells showed hyperplasia. Some cortical tissue broke down with the development of fibrous tissue containing cholesterol clefts and calcium deposits. In surviving transplants, growth stimulation of the cortical cells was indicated by the appearance of hyperplastic foci and cortical nodules. The latter were found in eight animals with grafts 8 to 18 months previous to death (mean age 14.6 months). The fate of the hypophyseal grafts has been described previously.[2a] A preserved graft is illustrated in Figure 2. The cytologic changes will be reported at a later date.

Changes in Adrenals.—The changes were graded as follows: The symbol O stands for no change; grade I indicates focal cortical hyperplasia; grade II denotes diffuse cortical hyperplasia; grade III designates the presence of nodular cortical tumors replacing parts of the adrenal cortex and medulla. The following photomicrographs (Fig. 3) show representative examples of the changes. The findings are tabulated in Table 1.

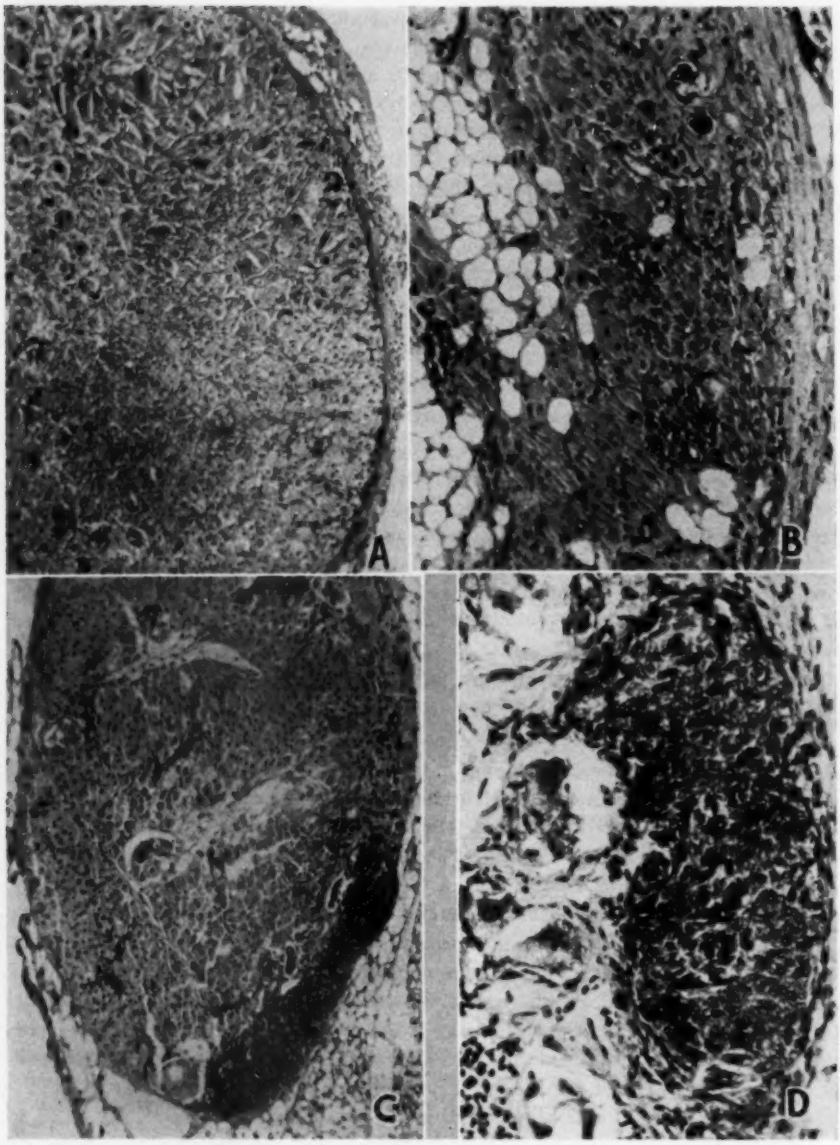

Fig. 1.—Sections through adrenal grafts. *A*, 2-month-old graft. The animal had also received anterior hypophyseal grafts. There is breakdown of cortical cells and formation of cholesterol clefts; × 100. *B*, 10-month-old graft. Much of the cortical tissue is preserved. Two small foci of calcification are seen at upper right; × 190. *C*, 17-month-old graft showing cortical hyperplasia; × 100. *D*, 10-month-old graft. The animal had also received anterior hypophyseal grafts. There is beginning nodule formation in the cortex; × 300.

Untreated controls (10 animals): In 40% of the older male animals examined at a mean age of 21 months, the adrenal cortex was resting. Growth stimulation grade I was present in 40% at a mean age of 20 months, and growth stimulation grade II was seen in 20% of the mice at a mean age of 22.5 months. Growth stimulation grade III was not observed.

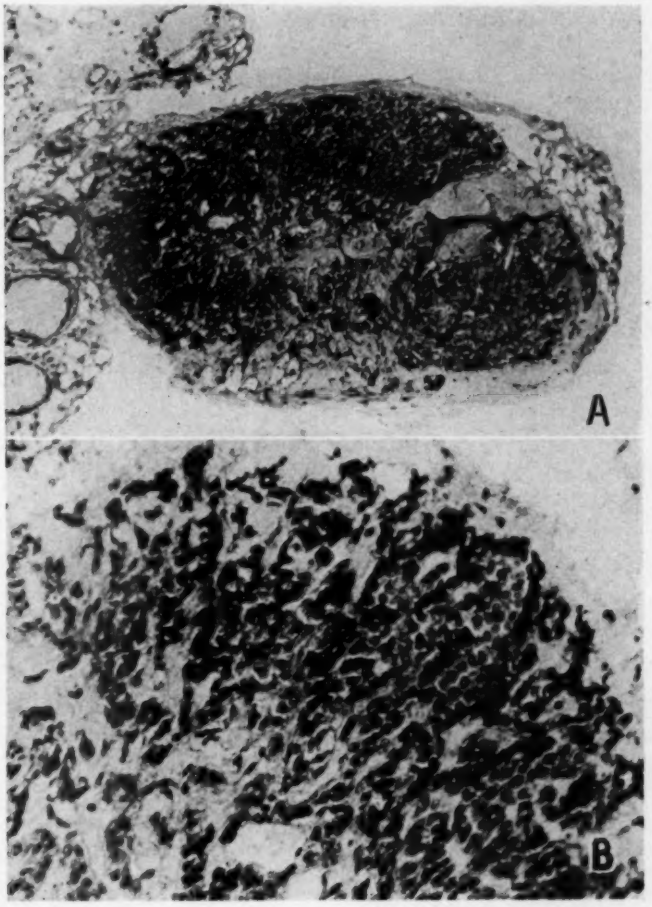

Fig. 2.—*A*, section through an anterior hypophyseal graft 18 months after transplantation showing preserved hypophyseal tissue. Mammary tissue (site of grafting) is seen at left; × 90. *B*, same as figure 2 *A*; × 300.

Castrates (36 animals): In 11.1% of these mice the adrenal cortex was resting (mean age 2.5 months). There was growth stimulation grade I in 33.3% of the mice at a mean age of 11.2 months, growth stimulation grade II in 13.9% of the animals at a mean age of 19.9 months, and growth stimulation grade III in 41.7% of the mice at a mean age of 20.1 months.

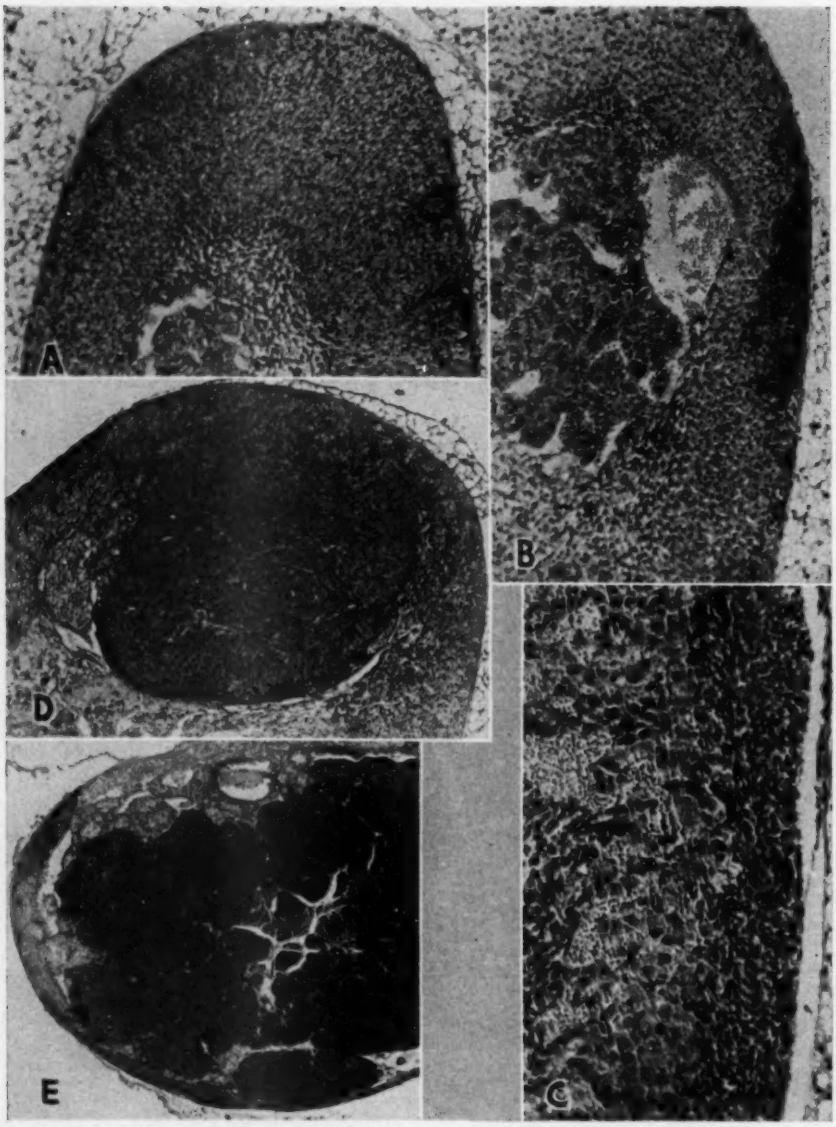

Fig. 3.—Sections through in situ adrenals. *A*, untreated control male, 20 months old. The cortex is resting; × 100. *B*, 9-month-old castrate bearing adrenal and hypophyseal grafts. The cortex shows focal hyperplasia (Grade I stimulation); × 100. *C*, 19-month-old castrate. The cortex shows diffuse hyperplasia (Grade II stimulation); × 200. *D*, 19-month-old castrate. A cortical adenoma is seen; × 30. *E*, 20-month-old castrate. The animal had received adrenal and hypophyseal grafts. A large cortical adenoma is seen; × 30.

Castrates Bearing Adrenal Grafts (35 animals): In 11.4% of these mice (mean age 4.5 months) the adrenals were resting. Growth stimulation grade I was noted in 34.3% of the animals (mean age 10.3 months); growth stimulation grade II was present in 14.3% (mean age 16.6 months), and growth stimulation grade III was observed in 40% of the animals (mean age 13.9 months).

Castrates Bearing Adrenal and Hypophyseal Grafts (37 animals): In 5.4% of the mice (mean age 5 months) the adrenals were resting. In 2.7% of the mice (mean age 19 months) growth stimulation grade I was seen. Growth stimulation

TABLE 1.—*Findings in the Adrenals in Various Experimental Groups*

		Changes, Grade							
		O		I		II		III	
Experiment	Total No. of Mice	%	Mean Age, Mo.; Range	%	Mean Age, Mo.; Range	%	Mean Age, Mo.; Range	%	Mean Age, Mo.; Range
Controls	10	40.0	21.0 (20-22)	40.0	20.0 (19-21)	20.0	22.5 (22-23)	0.0	....
Castrates	36	11.1	2.5 (2-3)	33.3	11.2 (5-21)	13.9	19.9 (19-21)	41.7	20.1 (21-23)
Castrates bearing adrenal grafts	35	11.4	4.5 (2-10)	34.3	10.3 (3-20)	14.3	16.6 (12-20)	40.0	13.9 (10-21)
Castrates bearing adrenal and hypophyseal grafts	37	5.4	5.0 (2-10)	2.7	19.0 (19)	27.0	9.1 (3-19)	64.9	14.3 (6-21)

TABLE 2.—*Findings in the Mammary Glands of Various Experimental Groups*

		Changes, Grade							
		O		I		II		III	
Experiment	Total No. of Mice	%	Mean Age, Mo.; Range	%	Mean Age, Mo.; Range	%	Mean Age, Mo.; Range	%	Mean Age, Mo.; Range
Controls	10	100.0	22.3 (19-23)	0.0	....	0.0	....	0.0	....
Castrates	36	58.3	12.6 (2-21)	25.0	16.5 (10-21)	13.9	19.8 (19-21)	2.8	21.0 (21)
Castrates bearing adrenal grafts	35	51.4	10.0 (2-20)	14.3	14.5 (7-20)	25.7	16.5 (13-21)	8.6	19.7 (19-21)
Castrates bearing adrenal and hypophyseal grafts	37	43.3	10.5 (2-18)	10.7	7.3 (2-14)	13.6	13.6 (9-19)	32.4	16.8 (9-21)

grade II was found in 27% (mean age 9.1 months), and growth stimulation grade III was observed in 64.9% of the mice (mean age 14.3 months).

Changes in Mammary Glands.—The findings were classified as follows: The symbol O is used to indicate a resting mammary gland; grade I denotes ductal hypertrophy with secretions and lateral budding; grade II indicates secretions with lateral and terminal budding of the ducts, and grade III designates the formation of acinar nodules. Details and representative photomicrographs have been published previously.[1] The data are presented in Table 2.

Untreated Controls: The mammary glands were resting in all animals, the mean age being 22.3 months.

Castrates: In 21 of 36 animals (58.3%) with a mean age of 12.6 months, the mammary glands were resting. Growth stimulation grade I was seen in nine mice

(25%) at a mean age of 16.5 months; growth stimulation grade II was present in five mice (13.9%) at a mean age of 19.8 months, and growth stimulation grade III was observed in one mouse (2.8%) at the age of 21 months.

Castrates Bearing Adrenal Grafts: In 18 out of a total of 35 mice (51.4%) with a mean age of 10 months, the mammary glands were resting. Growth stimulation grade I was seen in five mice (14.3%) with a mean age of 14.5 months; growth stimulation grade II was found in nine mice (25.7%) with a mean age of 16.5 months, and growth stimulation grade III was present in three mice (8.6%) with a mean age of 19.7 months.

Castrates Bearing Adrenal and Hypophyseal Grafts: In 16 of 37 mice (43.3%) with a mean age of 10.5 months, the mammary glands were resting. Growth stimulation grade I was observed in 4 mice (10.7%) with a mean age of 7.3 months; growth stimulation grade II was seen in 5 mice (13.6%) with a mean age of 13.6

TABLE 3.—*Correlation of Findings in Adrenal Cortices with Those in Mammary Glands*

Experiment	Mammary Growth, Grade	No. of Mice	% of Total	Incidence of Adrenocortical Changes, Grade			
				O	I	II	III
Castrates (36 mice)	O	21	58.4	19.0	42.9	9.5	28.6
	I	9	25.0	0.0	33.3	22.2	44.5
	II	5	13.8	0.0	0.0	20.0	80.0
	III	1	2.8	0.0	0.0	0.0	100.0
Castrates bearing adrenal grafts (35 mice)	O	18	51.5	22.2	38.9	16.7	22.2
	I	5	14.3	0.0	80.0	0.0	20.0
	II	9	25.6	0.0	11.1	22.2	66.7
	III	3	8.6	0.0	0.0	0.0	100.0
Castrates bearing adrenal and hypophyseal grafts (37 mice)	O	16	43.4	12.4	0.0	43.8	43.8
	I	4	10.8	0.0	0.0	50.0	50.0
	II	5	13.4	0.0	0.0	0.0	100.0
	III	12	32.4	0.0	8.3	8.3	83.4
Irrespective of experimental group (108 mice)	O	55	50.9	18.2	29.1	21.8	30.9
	I	18	16.7	0.0	38.9	22.2	38.9
	II	19	17.6	0.0	5.3	15.9	78.8
	III	16	14.8	0.0	6.3	6.3	87.4

months, and growth stimulation grade III was noted in 12 mice (32.4%) with a mean age of 16.8 months. The sex of the donors of the grafts was apparently without effect on mammary growth.

Comparison of Adrenal and Mammary Growth Stimulation.—In order to facilitate a comparison between the state of growth of the adrenals and that of the mammary glands, Table 3 was prepared.

If the findings in adrenals and mammary glands are considered, irrespective of the experimental group, the following results are obtained: Resting mammary glands were found in 55 of the total of 108 mice (50.9%). In 10 of these animals (18.2%) the adrenal cortex was resting; in 16 mice (29.1%) it showed focal hyperplasia and in 12 mice (21.8%) diffuse hyperplasia; in 17 mice (30.9%) adenomas were found. Apparently none of these 45 hyperplastic adrenals produced estrogen; or if they did, the amounts given off were not sufficient to promote mammary growth. Mammary growth stimulation grade I was present in 18 mice (16.7% of the total). Resting adrenals were absent in this group; seven mice (38.9%) showed focal hyperplasia, and four mice (22.2%) disclosed diffuse cor-

tical hyperplasia; seven mice (38.9%) had cortical adenomas. Mammary growth stimulation grade II was noted in 19 mice (17.6% of the total). Again none of the animals showed resting adrenal cortices; 1 mouse (5.3%) had focal hyperplasia; 3 mice (15.9%) had diffuse cortical hyperplasia, and 15 mice (78.8%) had developed cortical adenomas. Mammary growth stimulation grade III was observed in 16 of the total of 108 mice (14.8%). None of these mice showed resting adrenals, and only one animal each (6.3%) had focal or diffuse cortical hyperplasia, while the remaining 14 animals (87.4%) had cortical adenomas.

A comparison of the findings in the individual experimental groups gives the following results: Among 36 castrates, 21 mice (58.4%) showed resting mammary glands. Of these 21 mice, 4 (19%) had also resting adrenals; the adrenals of 9 animals (42.9%) showed focal hyperplasia; those of 2 animals (9.5%) showed diffuse hyperplasia, and those of the remaining 6 animals (28.6%) had cortical adenomas. Nine mice (25%) showed mammary growth stimulation grade I. Of these mice, none had resting adrenals, three mice (33.3%) had focal adrenocortical hyperplasia, and two (22.2%) had diffuse adrenocortical hyperplasia; four animals (44.5%) had cortical adenomas. Five castrates (13.8%) developed mammary growth grade II. One (20%) of these showed diffuse cortical hyperplasia, and four (80%) had cortical adenomas. Finally, one castrate (2.8) showed mammary growth grade III; cortical adenomas were present in the adrenals of this animal.

Among 35 castrates bearing adrenal grafts, the distribution of mammary and adrenal changes was similar to that seen in castrate controls, but mammary growth was somewhat more stimulated than in the latter. Eighteen mice (51.5%) had resting mammary glands. Of these 18 mice, 4 (22.2%) had unchanged adrenals; 7 (38.9%) showed focal cortical hyperplasia, and 3 (16.7%) had diffuse cortical hyperplasia, whereas 4 (22.2%) had cortical adenomas. Five mice (14.3%) showed mammary growth stimulation grade I. No resting adrenals were found in this group; four (80%) of the mice had focal hyperplasia, and one (20%) had cortical adenomas. Nine mice (25.6%) showed mammary growth stimulation grade II. None of these mice had resting adrenals; the adrenal of one mouse (11.1%) showed focal hyperplasia; those of two mice (22.2%) showed diffuse hyperplasia, and in six mice (66.7%) adenomas were found. Three of the animals (8.6%) disclosed mammary growth stimulation grade III. In all of these mice, cortical adrenal adenomas were found.

Among 37 castrates bearing adrenal and hypophyseal grafts, the percentage of mice with resting mammary glands had decreased from 58.4% in castrates and 51.1% in castrates bearing adrenals to 43.4%. Sixteen mice (43.4%) had resting mammary glands. Of these 16 mice, 2 (12.4%) had resting adrenals; 7 (43.8%) showed diffuse cortical hyperplasia, and in the remaining 7 (43.8%) cortical adenomas had developed. Four mice (10.8%) disclosed mammary growth stimulation grade I. Two (50%) of these showed diffuse cortical hyperplasia, and the other two (50%) had cortical adenomas. Five animals (13.4%) had mammary growth stimulation grade II, and all of these showed cortical adenomas. Finally, in 12 mice (32.4%) mammary growth stimulation grade III was noted. Of these, one each (8.3%) had focal or diffuse cortical hyperplasia, while in 10 mice (83.4%) cortical adenomas were found.

COMMENT

The adrenal cortices of male mice of strain A orchiectomized one to three days after birth showed hyperplasia varying in degree, including adenomas. These changes did not become conspicuous before the age of 19 months, while they are frequent and occur at an early age in castrates of strains Bagg albino, C3H, DBA, and CE.[3] Homeografts of adrenals did not noticeably influence the postcastrational changes in the animals' own adrenals; however, the grafts themselves underwent changes similar to those seen in the former. This is according to expectation since grafted adrenals have been shown to possess the same strain characteristic growth pattern as adrenals left in situ.[4] In the presence of adrenal grafts, mammary growth was intensified, probably owing to the action of estrogen given off by the grafted adrenals. Whether or not the latter also caused hypophyseal changes, as observed in castrates of other strains,[5] will be investigated. Anterior hypophyseal grafts considerably accelerated the formation and increased the number and size of hyperplastic cortical lesions in both the grafted and the animals' own adrenals.

The question now arises whether or not the hyperplastic adrenal cortices produce hormone, especially estrogen. If so, is there a direct effect on mammary growth, or is there at first a stimulation of the hypophysis which in turn induces mammary proliferation?

Since the salivary glands and the kidneys of our mice failed to show microscopic changes characteristic of masculinization, androgen was apparently not given off by the stimulated adrenals. Since, on the other hand, in each experimental group a number of mice (22.2 to 43.8%) showed resting mammary tissue in the presence of adrenal cortical adenomas, many of the latter must be regarded as nonfunctional, as far as the production of estrogen is concerned. However, acinar growth in the mammary gland was associated with cortical adenomas in all but two instances in which the adrenal cortex was only slightly or moderately stimulated.

The elevenfold increase in the number of mice showing acinar growth in the mammary glands of castrates bearing adrenal and hypophyseal grafts indicates the significance of growth-promoting secretions given off by the latter. This finding, together with the simultaneous increase in number and size of the adrenocortical lesions, admits of the following explanations: (1) The hypophyseal secretions may stimulate the adrenal cortices and the mammary glands independently; (2) the hypophyseal secretions may stimulate at first the adrenal cortex which subsequently releases increased amounts of estrogen; (3) both these mechanisms may be operating. Except in two instances, advanced mammary stimulation was seen only in the presence of accentuated adrenal growth. This fact suggests an increased output of estrogen by the adrenals in the presence of grafted hypophyses. The

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4. Huseby, R. A., and Bittner, J. J.: Differences in Adrenal Responsiveness to Postcastrational Alteration, as Evidenced by Transplanted Adrenal Tissue, Cancer Res. **11**:258, 1951.

5. Dickie, M. M., and Woolley, G. W.: Spontaneous Basophilic Tumors of the Pituitary Glands in Gonadectomized Mice, Cancer Res. **9**:372, 1949.

latter would act on both the in situ adrenals, as well as on the grafted ones, and thus considerably enhance the production of estrogen. This effect would resemble that exerted by hypophyseal grafts on in situ and on grafted ovaries.[6] It seems unlikely, however, that the increased output of estrogen would suffice to cause the observed increase in the number of mice showing acinar growth in the mammary glands. Hypophyseal secretions may exert an additional direct effect on mammary growth. This would be plausible in the light of previous results,[7] as well as in view of the fact that in the present series occasional acinar growth in the mammary gland was noted in the presence of only slightly or moderately stimulated adrenal cortices.

SUMMARY

Adrenal cortical hyperplasia of varying degree, including adenomas, was observed in male mice of strain A castrated one to three days after birth. Homeogenous adrenal grafts made in these castrates showed hyperplastic changes similar to those seen in the in situ adrenals. The hyperplastic lesions were increased in number and size, and their onset was accelerated in the presence of homeogenous anterior hypophyseal grafts. About 30% of the adrenocortical tumors failed to release either androgenic or estrogenic hormones. The remaining adrenal tumors were associated with mammary growth stimulation of varying degree. This mammary growth stimulation is thought to be due at least partly to the action of estrogen produced by the in situ, as well as the grafted, adrenals. There is evidence that an anterior hypophyseal hormone likewise exerts a growth-promoting effect on the mammary tissue.

6. Loeb, L., and Kirtz, M. M.: The Effects of Transplants of Anterior Lobes of the Hypophysis on the Growth and Development of the Mammary Gland and on the Development of Mammary Gland Carcinoma in Various Strains of Mice, Am. J. Cancer **36**:56, 1939. Huseby, R. A., and Bittner, J. J.: The Development of Mammary Cancer in Castrate A Strain Male Mice Bearing Ovarian Grafts, Cancer Res. **11**:450, 1951. Silberberg and Silberberg.[1]

7. Riddle, O.: Endocrine Aspects of the Physiology of Reproduction, Ann. Rev. Physiol. **3**:573, 1941. Mixner, J. P., and Turner, C. W.: The Mammogenic Hormone of the Anterior Pituitary: The Lobulo-Alveolar Growth Factor, Research Bulletin 378, University of Missouri Agricultural Experimental Station, 1943. Gardner, W. U., and White, A.: Mammary Growth in Hypophysectomized Mice, Anat. Rec. **82**:414, 1942. Silberberg and Silberberg.[1]

MECHANISM OF SOFTENING OF TUBERCLES

II. Behavior of Desoxyribonuclease in Tubercles Developing in the Lungs of Rabbits

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INTRODUCTION AND REVIEW OF THE LITERATURE [1]

THE PECULIAR type of cellular injury characteristic of infection with virulent tubercle bacilli has been known for a long time under the name of caseous necrosis.[2] However, this type of lesion has been vaguely defined only on the basis of the histological appearance, and up to the present the chemical processes concerned in its pathogenesis have not been adequately worked out. Lurie [3] demonstrated that in rabbits which have been infected with virulent bacilli caseation is usually observed about the third week of the disease, when the monocytes, which make up the bulk of the cellular material in the caseous area, have been transformed into epithelioid cells and allergy to tuberculin has developed. This suggests that caseation is associated with some degree of acquired resistance to infection with tubercle bacilli. Lurie [4] later showed that estrogen, which retards the progress of tuberculosis at the portal of entry in the skin and diminishes its dissemination to the internal organs in highly inbred animals chiefly by reducing the permeability of the connective tissue, may increase the extent of caseation in animals with progressive tuberculosis. Cortisone has the reverse effect on caseation. It also reduces capillary permeability, enhances the growth of tubercle bacilli within macrophages, diminishes antibody production, and suppresses nonspecific and allergic inflammatory processes.[5]

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1. The literature on this subject has been reviewed up to 1932 by Wells, H. G., and Long, E. R.: The Chemistry of Tuberculosis, Ed. 2, Baltimore, Williams & Wilkins Company, 1932. We shall therefore limit ourselves to publications which have appeared during the past 20 years.

2. Rich, A. R.: The Pathogenesis of Tuberculosis, Ed. 2, Springfield, Ill., Charles C Thomas, Publisher, 1951.

3. Lurie, M. B.: J. Exper. Med. **57**:181, 1933.

4. Lurie, M. B.; Abramson, S., and Allison, M. J.: Am. Rev. Tuberc. **59**:168, 1949.

5. Lurie, M. B.; Zappasodi, P.; Dannenberg, A. M., Jr., and Swartz, I. B.: Science **113**:234, 1951. Lurie, M. B.; Zappasodi, P.; Dannenberg, A. M., Jr., and Lynch, E. C.: Tr. Nat. Tuberc. A. **47**:47, 1951.

Employing the ear chamber technique and the motion picture camera, Ebert and others [6] followed the first stages in the development of caseation in rabbits. They observed stasis and localized thrombosis of small venules surrounding dense collections of cells (monocytes, lymphocytes, macrophages, and some polymorphonuclear cells, as well as giant cells). At that time the tuberculin reaction was positive, and histological sections showed tubercles which had undergone extensive caseous necrosis.

Hess [7] offers the following hypothesis concerning the pathogenesis of caseation. Infection with virulent tubercle bacilli causes liberation from the blood plasma of an exudate rich in phagocytic cells and fibrinogen. An unknown toxic factor in the tubercle bacillus leads to desquamation and necrosis of many of these cells. Fibrin precipitates out from the exudate, undergoes swelling, loses its capacity to stain properly, is digested by a trypsin-like ferment, and becomes converted into a substance known as fibrinoid. The exudative cells which are trapped in the meshwork of this fibrinoid mass become necrotic, either because of a failure of osmotic exchange resulting in toxic injury or perhaps owing to coagulation of the protoplasm of the cell. This question is not yet settled. The suddenness of the formation of fibrinoid explains why alveoli are filled simultaneously with fibrinoid and with unchanged fibrin.

The author was able to prepare a glycerin extract of caseous tuberculous tissue which transforms native fibrin into fibrinoid. This substance has its optimal activity between pH 5 and 6; it is thermolabile, nondialyzable, and not precipitated by means of 50% $MgSO_4$. It is not found in old tuberculin (OT) or in extracts of tubercle bacilli but can be extracted from the serum of tuberculous guinea pigs six weeks after infection.

Employing a silver impregnation method, Fresen [8] showed that a reticular fibrous structure is pathognomonic of the tubercle. In caseous necrosis, only local argyrophile fibers are found, whereas the epithelioid cells which are formed in the periphery develop their own reticulum. The exudative caseating phase of the tuberculous inflammatory process is accompanied by destruction of the preexisting reticulin network. The author concludes that caseous necrosis which develops during the primary exudative process precedes the formation of tubercles.

Murith,[9] employing a similar silver impregnation technique, observed that the organization of the caseous mass is associated with the penetration of reticulum fibers from the surrounding granulation tissue. These fibers are formed by the enzymatic action of fibrocytes, are connected with the peripheral collagenous fibers, and are subsequently transformed into collagenous fibers. A further advance of the caseation indicates a new inflammatory reaction.

Another approach to the study of the pathogenesis of caseation necrosis was that of Otero and others.[10] Working with white rats, which are usually resistant to

6. Ebert, R. H.; Ahern, J. J., and Bloch, R. G.: Proc. Soc. Exper. Biol. & Med. **68**:625, 1948.

7. Hess, W.: Schweiz. Ztschr. Path. u. Bakt. **10**:260, 1947.

8. Fresen, O.: Klin. Wchnschr. **28**:194, 1950; abstracted, Excerpta Med. V **5**:416, 1952; Beitr. klin. Tuberk. **104**:104, 1950; abstracted, Excerpta Med. V **5**:3378, 1952.

9. Murith, G.: Schweiz. Ztschr. Tuberk. **8**:99, 1951.

10. Otero, P. M.; Koppisch, E., and Axtmayer, J. H.: Puerto Rico J. Pub. Health **9**:314, 1934.

tuberculous infection and in which allergy to tuberculin is not demonstrable, they found that caseation developed only in those animals which had been totally depleted of vitamin A and infected intraperitoneally with avian (but not with human or bovine) tubercle bacilli. Unfortunately, the authors did not employ the method of airborne infection.

Brieger [11] employed chick embryos and noted that, after intravenous injection of tubercle bacilli, caseation took place in the centers of foci, in spite of the fact that hypersensitiveness is not likely to develop under these experimental conditions. We thus have contradictory evidence as to the relation between the development of caseation and allergy. This subject, therefore, remains for further investigation.

Several attempts have been made to reproduce the lesions of tuberculous caseous necrosis in animals by means of chemical substances fractionated from tubercle bacilli. The first was that of Sabin, Doan, and Forkner,[12] who employed Anderson's phosphatide A3, which unfortunately was not free from bacillary debris.[13] More recently Ungar and others [14] used synthetic methyl-substituted long chain fatty acids closely related to the naturally occurring phthioic acid. The characteristic lesions which developed after intraperitoneal injection into guinea pigs resembled those due to phthioic acid. They were "necrosis, abscess formation and foam cell nodules which may exhibit the features of a foreign body or a tuberculoid granuloma." Lederer and others [15] produced somewhat similar lesions by injecting subcutaneously into guinea pigs a lipopolysaccharide extracted from tubercle bacilli. Whether or not the experimental conditions employed and the cytology of the lesions described by these authors can be regarded as satisfying all the essential criteria of disseminating tuberculous caseous necrosis remains to be determined.

One of the unique characteristics of the tuberculous caseous lesion, in contrast with other types of necrotic tissue, is its marked delay in undergoing autolysis or softening. The reasons for this delay are still unknown. In order to throw light upon this question it would be desirable to follow the changes which occur in the essential chemical constituents of the tubercle as it undergoes calcification and healing, on the one hand, or softening (liquefaction), on the other. Unfortunately, data of this kind are scanty, and what little there are in the older literature were acquired by methods which today are open to criticism. This is especially true in regard to the proteins. It is known from the work of Schmoll [16] that caseous tissue contains coagulated albumin but no soluble protein. Several years ago, Lurie showed that "caseous tissue of a tuberculous rabbit behaves like heterologous, not homologous, protein and stimulates the formation of precipitins. The latter are not antibodies to the bacilli present but are specifically related to the protein debris, which must

11. Brieger, E. M.: Advances in Tuberculosis Research, New York, S. Karger, 1951, Vol. 4, p. 236.

12. Sabin, F. R.; Doan, C. A., and Forkner, G. E.: J. Exper. Med. (Supp. 3) **52**:18, 1930.

13. Boissevain, C. H., and Ryder, C. T.: Am. Rev. Tuberc. **24**:751, 1931; cited by Ungar, J.; Coulthard, C. E., and Dickinson, L.: Brit. J. Exper. Path. **29**:322, 1948.

14. Ungar, J.; Coulthard, C. E., and Dickinson, L.: Brit. J. Exper. Path. **29**:322, 1948.

15. Delaunay, A.; Asselineau, J., and Lederer, E.: Compt. rend. Soc. biol. **145**:650, 1951; Asselineau, J., and Lederer, E.: Experientia **7**:281, 1951; Husseini, H., and Elberg, S.: Cellular Reactions to Phthienoic Acid and Related Branched-Chain Acids, Am. Rev. Tuberc. **65**:655, 1952.

16. Schmoll, E.: Deutsches Arch. klin. Med. **81**:163, 1904.

represent modified tissue protein."[17] Hirszfeld and Halber[18] demonstrated that in caseous material there is an antigen similar to one present in pus, in circulating leucocytes, in infarcts, and in cancerous tissue. D'Alessandro and Paternostro[19] pointed out that alterations in lipids, occurring during various disease processes, may be responsible for these crossed serologic reactions. Further investigation of the proteins of caseous material by modern immunochemical methods is needed.

As shown by Caldwell[20] and others, lipids constitute a prominent feature in the chemical anatomy of the caseous tubercle. Hess,[7] however, regards these as merely incidental to the rapid death of cells. The studies of Caldwell were extended by Cova,[21] who observed an increase in the free and bound cholesterol and a decrease in phospholipids but no change in the neutral fats and fatty acids of caseous material from bovine tuberculous lungs. There was also a striking increase in the unsaponifiable substance other than cholesterol but no change in the iodine numbers of the fatty acids, in the saponification number, or in the esterification of the total fat. Similar observations were made by Weiss and Schultz[22] on rabbits which had been infected experimentally with virulent bovine, Ravenel tubercle bacilli by the intratracheal route. In animals which were killed 16 weeks after primary infection or 5 weeks after reinfection and which showed gross evidence of diffuse caseous infiltration of the lungs, there were significant increases in the total cholesterol content of these tissues.

Grundland and others[23] speculate that the fats of the caseous matter may originate from the fusion of the protoplasmic fats with the lipids of the tubercle bacillus. Since the latter cannot be transformed by oxidation during their prolonged contact with the tissues, they exert a stronger affinity than that of the proteins toward the fats of the protoplasm which are dispersed in the lipoproteic complexes.

Lipases which are concerned in the hydrolysis of various lipid substances are a prominent feature in the current literature on tuberculosis. Scoz[24] analyzed bovine tuberculous material from the slaughter house and noted the presence of a tributyrinase which hydrolyzed triolein optimally at pH 9, but he presented no quantitative data as to its distribution in various parts of the caseous tubercle. Prina[25] infected guinea pigs intratracheally with human tubercle bacilli and by means of Gomori's histochemical technique demonstrated lipase in the central areas of tubercles. Similar observations were made by Frahm and others.[26]

17. Lurie, M. B., cited by Long, E. R.: Arch. Path. **28**:719, 1939.

18. Hirszfeld, L., and Halber, W.: Ztschr. Immunitätsforsch. **88**:41, 1935; Klin. Wchnschr. **16**:878, 1937.

19. D'Alessandro, G., and Paternostro, C.: Boll. Soc. ital. biol. sper. **20**:483, 1945.

20. Caldwell, G. T.: J. Infect. Dis. **24**:81, 1919.

21. Cova, N.: Gior. ital. tuberc. **2**:361, 1948.

22. Weiss, C., and Schultz, J.: U. S. Public Health Service, Report to Tuberculosis Study Section, April 1, 1949, unpublished data.

23. Grundland, I., and Luzzati, D.: Compt. rend. Soc. biol. **143**:981, 1949. Grundland, I.; Bulliard, H., and Maillet, M.: Presse méd. **59**:406, 1951.

24. Scoz, G.: Ann. Ist. Carlo Forlanini **7**:247, 1943; abstracted, Chem. Abstr. **42**:5476i, 1948.

25. Prina, C.: Boll. Soc. ital. biol. sper. **27**:1071, 1951.

26. Frahm, H.; Lembke, A., and Schmidt, H.: Zentralbl. Bakt. **156**:400, 1951.

Studies have also been reported on the distribution of alkaline phosphatases which liberate orthophosphoric acid from compounds of various types.[27] Horii [28] records the presence of this enzyme in the peripheral zones of tubercles but its absence in the caseous centers. Similar observations were made by Takeuchi and Takamatsu [29] and by Prina.[25] Grogg and Pearse [30] injected bovine tubercle bacilli subcutaneously into guinea pigs and rabbits. In the latter, the necrotic centers of lesions contained no acid phosphatase, while the mononuclear phagocytes contained a moderately strong concentration of this enzyme. "Alkaline phosphatase usually occurred only in the cytoplasm of polymorphs and in the vascular endothelia within the lesions, but free alkaline phosphatase was present in the necrotic areas. Single groups of mononuclear phagocytes within the lesions gave a strong positive reaction for esterase in their cytoplasm."

The results observed when tuberculous lesions of rabbits were stained by the method for 5-nucleotidase were very similar to those obtained by the staining method for alkaline phosphatase.

Among studies on proteolytic enzymes of caseous material are those of Frahm and others.[26] They demonstrated that, while normal guinea pig lung tissue contains no proteinase which can hydrolyze casein, this enzyme becomes activated in animals which have been infected with human tubercle bacilli or in homogenates of normal guinea pig lung in which tubercle bacilli have grown in vitro.

In the work of Weiss and Boyar-Manstein,[31] tubercles were produced in the lungs of rabbits by means of the Wells apparatus for air-borne infection. The animals were killed at varying intervals of time, thus permitting observations during successive stages in the development of discrete tubercles. There was evidence of greatly increased activity of the enzymes benzoyl-l-arginine amidase and leucine amino peptidase in the inflammatory zones of the tubercles. The areas of lung intermediate between primary tubercles, which contained only microscopic areas of cellular infiltration, showed only a moderate increase in the rate of enzymatic hydrolysis. In the centers of the tubercles, where there was gross evidence of caseation, the rate of substrate cleavage was markedly decreased. The rate of hydrolysis of leucine amino peptidase became very low, and that of benzoyl-l-arginine amidase reached zero in areas where macroscopic softening had developed. The data suggest that wherever there are intact cells there is proteinase action and wherever the cells have undergone complete necrosis or softening there is a decrease or absence of enzymatic hydrolysis.

PLAN OF INVESTIGATION

Having demonstrated a pattern of distribution of two proteinases in experimentally induced tubercles and particularly the reduction or disappearance of these enzymes in the contents of softened tubercles, we then proceeded to make similar investigations on the enzyme desoxyribonuclease, since this enzyme is concerned in

27. Roche, J., in The Enzymes: Chemistry and Mechanism of Action, edited by J. B. Sumner and K. Myrbäck, New York, Academic Press, Inc., 1950, Vol. 1, Pt. 1, p. 475.

28. Horii, T.: Arb. dritten Abt. anat. Inst. kaiserlich. Univ. Kyoto, Series C, No. 4, p. 49, 1939; cited by Frahm, and others.[26]

29. Takeuchi, T., and Takamatsu, H.: Tr. Soc. path. jap. **29**:490, 1939, cited by Frahm, and others.[26]

30. Grogg, E., and Pearse, A. G. E.: Brit. J. Exper. Path. **33**:567, 1952.

31. Weiss, C., and Boyar-Manstein, M. L.: Am. Rev. Tuberc. **63**:694, 1951.

the first stages of the depolymerization or breakdown of the giant fibers of desoxyribonucleic acid to nucleotide units.[32] This line of investigation seemed advisable since recent workers have called attention to the importance of nucleic acid in various normal and pathologic activities of the cell.[33] Among the studies reported are those on viral[34] and protozoan infections,[35] tumor formation,[36] and cellular necrosis.[37]

Of special interest to us are the histochemical researches of Monaci and Coppitz.[38] They observed an increase of cytoplasmic ribonucleic acid in cells of tuberculous granulomata. Desoxyribonucleic acid was abundant in the nuclei of lymphocytes, less abundant in those of Langhans' giant cells, and scarce in the nuclei of epithelioid cells. Ribonucleic acid had completely disappeared from areas of caseous necrosis, but desoxyribonucleic acid remained. The authors suggest that histones, denatured proteins, and glycoprotein compounds are liberated during the splitting of nucleoproteins. Similar observations were recorded by Bunting[39] on human autopsy material. He pointed out that "in tuberculous caseation, detectable desoxyribonucleic acids remain for what must be relatively long periods of time, since they are found in the midst of caseous tissue; this is not wholly unexpected since tuberculous tissue is non-vascularized."

MATERIALS AND METHODS

The method for producing experimental air-borne infection in rabbits by means of the Wells apparatus for quantitative droplet nuclei infection, which was employed in these studies, has been described by Wells, Ratcliffe, and Crumb[40] and by Lurie and others.[41] Eighteen animals were exposed at one session in groups of six. The advantages of this procedure and other technical details have been reported in a previous publication from this laboratory.[31] One part by weight of tissue from various areas of discrete tubercles was mixed with four parts of sterile distilled water and ground in a Potter homogenizer which was immersed in an ice bath. The homogenates were centrifuged in a refrigerated International centrifuge in order to obtain clear preparations for viscosimetric study.

Viscosimetric Procedures and Calculations.—The rate of depolymerization of desoxyribonucleic acid was determined by a viscosimetric method.[42] Into an Ostwald viscosimeter (water time, 16 to 18 seconds) was placed 2.5 ml. of a 0.1% highly polymerized desoxyribonucleic acid[43] dissolved in M/40 veronal acetate (Michaelis,[44] universal) buffer of suitable pH and

32. Davidson, J. N.: The Biochemistry of the Nucleic Acids, London, Methuen & Co., Ltd., 1950.

33. Sherry, S.; Tillett, W. S., and Christensen, L. R.: Proc. Soc. Exper. Biol. & Med. **68**:179, 1948. Tillett, W. S.; Sherry, S., and Christensen, L. R.: Proc. Soc. Exper. Biol. & Med. **68**:184, 1948. Harris, T. N., and Harris, S.: J. Exper. Med. **90**:169, 1949. Davidson.[32]

34. Bauer, D. J.: Brit. J. Exper. Path. **32**:7, 1951.

35. Lewert, R. M.: J. Infect. Dis. **91**:125, 1952.

36. Stowell, R. E.: Cancer **2**:121, 1949.

37. Klemperer, P.; Gueft, B.; Lee, S. L.; Leuchtenberger, C., and Pollister, A. W.: Arch. Path. **49**:503, 1950.

38. Monaci, M., and Coppitz, A.: Arch. "de Vecchi" anat. pat. **13**:1057, 1949.

39. Bunting, H.: Yale J. Biol. & Med. **22**:521, 1950.

40. Wells, W. F.; Ratcliffe, H. L., and Crumb, C.: Am. J. Hyg. **47**:11, 1948.

41. Lurie, M. B.; Heppleston, A. G.; Abramson, S., and Swartz, I. B.: Am. Rev. Tuberc. **61**:765, 1950.

42. (*a*) Laskowski, M., and Seidel, M. K.: Arch. Biochem. **7**:465, 1945. (*b*) Tillett, W. S., and Sherry, S.: J. Clin. Invest. **28**:173, 1949. (*c*) McCarty, M.: J. Exper. Med. **90**:543, 1949.

43. Desoxyribonucleic acid was purchased from Worthington Biochemical Laboratories, Freehold, N. J.

44. Michaelis, L.: Biochem. Ztschr. **234**:139, 1931.

containing M 0.003 $MgSO_4$ as activator when desired. Two-tenths milliliter of homogenate was added, and viscosimetric readings were made every five minutes at 30 C.; the last reading was not more than 30 minutes after the zero time.

The constant K of the reaction was calculated from the formula of Laskowski and Seidel,[42a]

$$K = \frac{1}{t} \log \frac{\eta_0}{\eta_t}.$$

This assumes that at any given time of incubation (t) the relative viscosity (η) of the reacting

TABLE 1.—*Comparative Activity of Desoxyribonuclease in Homogenates of Normal Rabbit Lung Tissue at Different pH Levels*

pH	Time Intervals of Observation, Min.	Relative Viscosity, η		$K \times 10^2$
5.0	0	3.00		.....
	5	2.76		0.724
	10	2.49		0.809
	15	2.29		0.782
	20	2.07		0.806
			Average:	0.780
5.5	0	3.06		.....
	5	2.72		1.080
	10	2.22		1.420
	15	1.90		1.390
	20	1.62		1.350
			Average:	1.330
6.0	0	4.12		.....
	5	3.81		0.680
	10	3.43		0.796
	15	3.09		0.833
	20	2.73		0.895
			Average:	0.801
6.5	0	4.30		.....
	5	4.08		0.456
	10	3.89		0.435
	15	3.73		0.412
	20	3.57		0.404
			Average:	0.427
7.0	0	4.19		.....
	5	4.14		0.104
	10	4.08		0.115
	15	4.02		0.120
	20	3.98		0.112
			Average:	0.123
7.5	0	4.38		.....
	5	4.33		0.100
	10	4.31		0.070
	15	4.24		0.094
	20	4.18		0.120
			Average:	0.096

mixture is proportional to the concentration of unattacked desoxyribonucleic acid and that depolymerization of desoxyribonucleic acid follows the equation of a first-order reaction. When comparing the activities of normal homogenates with those obtained from pathological material, we used the K values, as well as the time required to effect a given decrease in viscosity, as suggested by Oscar Bodansky.[45]

45. Bodansky, D.: J. Biol. Chem. **120**:555, 1937.

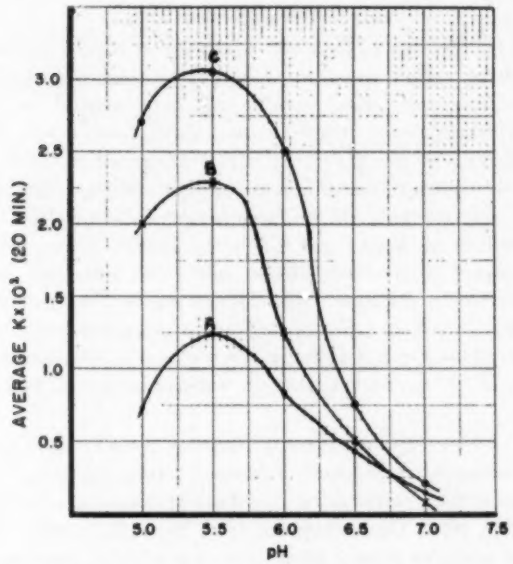


Chart 1.—The pH activity curve for desoxyribonuclease of homogenates prepared from normal and tuberculous lung tissue. *A*, normal; *B*, caseous material from centers of tubercles; *C*, inflammatory zones of tubercles.

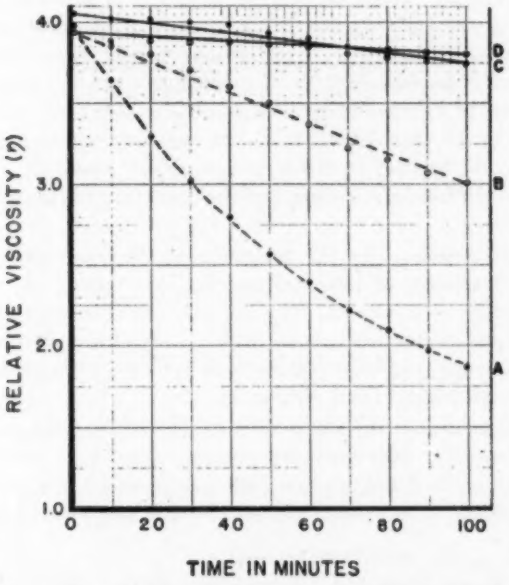


Chart 2.—Inhibitory effect of citrate ions upon desoxyribonuclease of lung homogenates. *A* and *C* indicate normal lung, without and with citrate, respectively. *B* and *D* indicate contents of softened tubercles, without and with citrate, respectively. Concentration of citrate equals 0.012 M.

EXPERIMENTAL RESULTS

Optimal pH for Activity of Desoxyribonuclease of Rabbit Tissue Homogenates.—After preliminary experimentation, it became evident that, unlike bovine pancreatic desoxyribonuclease, which works best in a neutral or slightly alkaline reaction,[46] desoxyribonuclease of rabbit tissues (lung, liver, spleen, and kidney) has practically no activity in this pH range. Data presented in Table 1 and Chart 1 show that the desoxyribonuclease of normal and tuberculous rabbit lung tissue works best in a weakly acid reaction, the optimum being at about pH 5. This is in accord with the publications of Maver and Greco[47]; Siebert, Lang, and Corbet,[48] and others.[49] This tissue desoxyribonuclease, moreover, does not require Mg^{++} as activator. Citrate ions in the form of the sodium salt in a concentration of 0.012 M inhibit its activity (Chart 2). Since solutions of desoxyribonucleic acid showed a tendency to precipitate at pH 5 in the presence of tissue homogenates, our tests for the distribution of desoxyribonuclease in various areas of tubercles were run at pH 6.

Distribution of Desoxyribonuclease in Various Areas of the Tubercle.—As seen from Table 2, homogenates prepared individually from the lungs of each of eight normal rabbits gave fairly uniform rates of desoxyribonuclease activity, the mean K value being 0.57×10^2. Those prepared from the inflammatory zones of experimentally induced tubercles showed about twice this activity, the mean K value being 1.08×10^2. Caseous necrotic material from the centers of tubercles, which on microscopic examination showed a variable number of intact cells of inflammatory origin, manifested varying degrees of enzymatic activity, the mean K value being 1.16×10^2. However, when the contents of softened tubercles were examined, they presented a striking reduction in, or absence of, desoxyribonuclease activity (mean K value $= 0.17 \times 10^2$). Comparisons based upon values for t (time required to cause a decrease of 0.5 unit in relative viscosity) gave similar results. Nitrogen analyses of softened material showed only about half the values for normal lung tissue or for that derived from inflammatory zones of tubercles. This is in harmony with the findings of earlier investigators.[1] However, we found no correlation between the nitrogen content and the desoxyribonuclease activity of tissue homogenates.

Experimental Evidence for the Existence of Enzyme Inhibitors in Caseous Tubercles.—I. Inhibition of Desoxyribonuclease in Vitro: (*a*) Direct evidence. When a homogenate was prepared from the contents of caseous or softened tubercles, and an aliquot portion was mixed with a homogenate of normal or inflamed tuberculous lung tissue, the desoxyribonuclease activity of the latter was reduced to 50%, or less, of its original value (Table 3).

(*b*) Indirect evidence; the effect of dilution. When homogenates of caseous material were tested for their desoxyribonuclease activity in serial dilutions (1:4, 1:8, 1:16), the time required to cause a decrease of half a unit in relative viscosity was not proportional to the dilution of enzyme but was much greater (Table 4).

46. Kunitz, M.: J. Gen. Physiol. **33**:363, 1950.

47. Maver, M. E., and Greco, A. E.: J. Biol. Chem. **181**:861, 1949.

48. Siebert, G.; Lang, K., and Corbet, A.: Biochem. Ztschr. **320**:418, 1950.

49. Brown, K. D.; Jacobs, G., and Laskowski, M.: J. Biol. Chem. **194**:445, 1952. Allfrey, V., and Mirsky, A. E.: J. Gen. Physiol. **36**:227, 1952.

TABLE 2.—*Viscosimetric Changes in Solutions of Desoxyribonucleic Acid Induced by Homogenates of Normal Rabbit Lung Tissue and Various Parts of Tubercles of Rabbit Lung*

Rabbit No.	Nitrogen (Mg./Ml. Homogenate)	$K \times 10^3$	Statistical Analysis of Data †	t * (Sec.)
		Normal Rabbit Lung Tissue		
302	2.32	0.422	Mean, $K \times 10^3 = 0.569$	16.4
306	2.42	0.861		11.0
310	2.12	0.468	$\sigma = 0.196$	14.5
349	2.55	0.507		13.0
313	2.31	0.621	S. E. = 0.069	14.0
402	2.74	0.785		11.0
334	2.17	0.452		16.0
339	2.88	0.437		16.0
	Average: 2.40			Average: 14.0
		Inflammatory Zones Surrounding Tubercles		
346	2.92	1.759	Mean, $K \times 10^3 = 1.075$	4.5
282	2.00	1.113		8.5
500	2.48	1.653	$\sigma = 0.451$	5.0
491	2.96	1.615		5.5
491	2.72	1.465	S. E. = 0.106	6.0
240	3.12	1.865		5.0
375	2.60	0.810	P ‡ = <0.01	10.0
338	2.08	0.865		9.0
371	2.59	0.387		16.0
374	2.13	0.921		8.0
250	1.82	0.793		11.0
254	3.20	1.358		6.5
314	2.34	1.047		7.5
286	2.01	0.428		17.0
345	2.62	0.777		9.5
272	2.44	0.855		9.0
272	2.44	1.006		7.5
345	2.02	0.564		13.5
	Average: 2.50			Average: 8.8
		Caseous Material in Centers of Tubercles		
346	2.05	1.442	Mean, $K \times 10^3 = 1.161$	5.5
282	1.80	1.235		6.5
500	2.46	1.640	$\sigma = 0.595$	4.5
260	1.84	0.801		9.0
493	2.77	2.505	S. E. = 0.171	2.3
375	2.19	0.440		16.0
338	2.12	0.687	P ‡ = <0.01	11.0
371	2.26	0.965		8.5
374	1.50	0.622		12.0
272	1.88	0.820		10.0
345	2.64	1.806		4.5
345	2.64	0.964		8.5
	Average: 2.18			Average: 8.2
		Contents of Softened Tubercles		
493	6.78	0.663	Mean, $K \times 10^3 = 0.168$	12.2
499	0.82	0.313		22.0
11	0.80	0.000	$\sigma = 0.195$	No activity
380	0.87	0.000		No activity
207	1.45	0.078	S. E. = 0.062	Very slight activity
495	1.17	0.235		27.0
229	0.98	0.115	P ‡ = <0.01	30.0
26	0.98	0.000		No activity
204	....	0.136		Very slight activity
496	....	0.142		Very slight activity
	Average: 1.00			

* t indicates time required to cause a decrease of 0.5 unit in relative viscosity (η).

† For statistical analyses and symbols, refer to Paterson, D. D.: Statistical Technique in Agricultural Research: Simple Exposition of Practice and Procedure in Biometry, New York, McGraw-Hill Book Company, Inc., 1939.

‡ P values are based upon comparisons with data on normal lung tissue.

This phenomenon suggests the presence of an inhibitor.[45] It was not observed with homogenates prepared from normal rabbit lung, from inflammatory zones of tubercles, or from areas of tuberculous lung which grossly showed no evidence of caseation.

TABLE 3.—*Evidence of the Presence of a Desoxyribonuclease Inhibitor in Caseous Material and in Contents of Softened Tubercles*

	Time Required to Produce Decrease in η *		
	Control Time, Min.	Enzyme Plus Inhibitor Time, Min.	Inhibition, %
Caseous Material			
1.0 unit decrease in η	6.5	10.0	53
1.3 unit decrease in η	10.5	19.0	81
		Average:	67
Contents of Softened Tubercles			
0.5 unit decrease in η	4.5	7.0	55
0.75 unit decrease in η	8.0	11.7	46
		Average:	50

* Refers to formula of Laskowski and Seidel [120]: $K = \frac{1}{t} \log \frac{\eta_0}{\eta_t}$.

TABLE 4.—*Evidence of the Presence of a Desoxyribonuclease Inhibitor in Caseous Tubercles*

Homogenate	Dilution	Time (Min.) to Cause Decrease of 0.5 Unit η	1.0 Unit η	Remarks
Caseous material from centers of tubercles	1:4	6.5	12.0	Inhibition
	1:8	20.0	35.0*	Inhibition
	1:16	25.0	40.0	Inhibition
Caseous material from centers of tubercles	1:4	5.0	13.0	Strong inhibition
	1:8	14.0	Slight activity	Strong inhibition
	1:16	Slight activity	No activity	Strong inhibition
Inflammatory zones surrounding caseous tubercles	1:4	7.0	12.5	No inhibition
	1:8	13.0	26.0	No inhibition
Inflammatory zones surrounding caseous tubercles	1:4	4.8	9.2	No inhibition
	1:8	10.0	19.0	No inhibition
Grossly uninvolved (but infected) lung tissue	1:4	1.0	20.0	No inhibition
	1:8	22.0	35.0	No inhibition
	1:16	33.0	45.0	No inhibition

* Interpolated from a graph.

II. Histochemical Evidence: By applying the Feulgen stain to sections of tissue removed from various areas of tubercles and adjacent lung tissue, it was observed that desoxyribonucleic acid was abundant in caseous material in spite of the presence of large concentrations of desoxyribonuclease and of proteinases. There were only occasional, scattered, minute remnants of desoxyribonucleic acid in the contents of softened tubercles. Similar observations were reported by Monaci and Coppitz [38] and by Bunting,[39] referred to above.

III. Inhibition of Proteinase by Contents of Caseous Tubercles: Material removed from caseous or softened tubercles also exerts inhibitory action on the

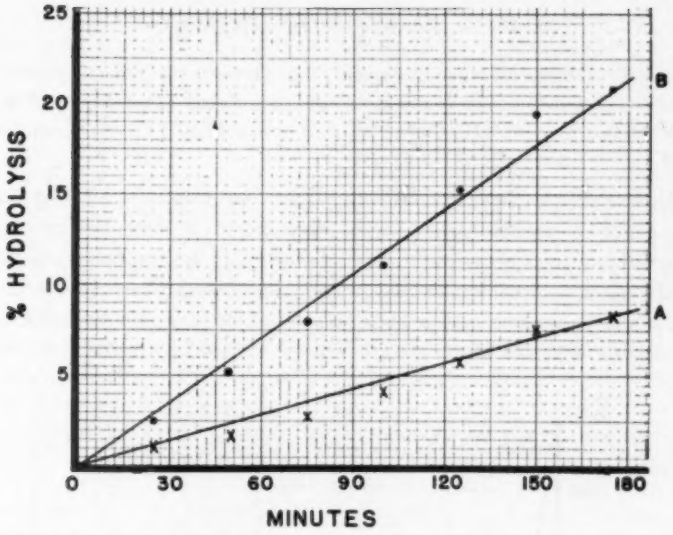


Chart 3.—Hydrolysis of benzoyl-l-arginine amide by homogenates of tuberculous rabbit lung tissue in presence and absence of material from softened tubercles. *A* and *B* indicate hydrolysis of substrate, with and without inhibitor, respectively.

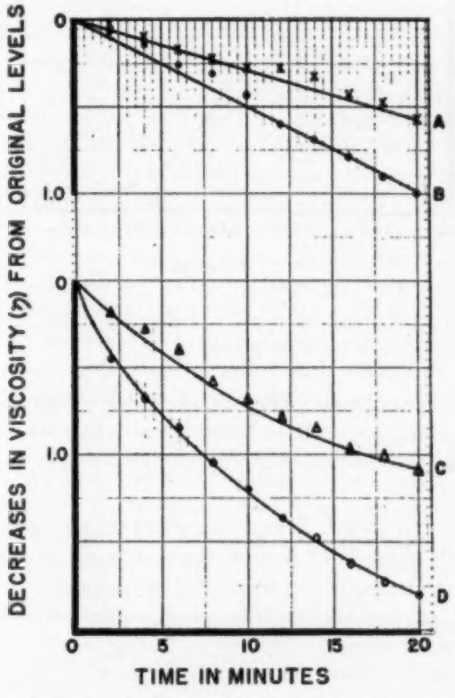


Chart 4.—Presence of desoxyribonuclease inhibitor in a cell-free extract prepared from a virulent Ravenel strain of tubercle bacilli. *A* and *B* indicate homogenate of normal rabbit lung tissue, with and without inhibitor, respectively. *C* and *D* indicate caseous material from tubercles, tested in a similar way.

endocellular proteolytic enzyme cathepsin II or benzoyl-l-arginine amidase. This inhibition is not selective but is effective against enzyme extracted from a variety of rabbit tissue—lung, liver, kidney. It is not neutralized by the addition of an excess of cystein hydrochloride (Chart 3).

IV. Evidence from Experimental Observations on Autolysis: That an enzymatic inhibitor exists in caseous tissue is also suggested by in vitro studies on aerobic autolysis which are in progress in this laboratory.[50] In contrast with the fairly active autolysis of normal rabbit lung tissue and of lung tissue which is situated between tubercles, caseous tissue autolyzes very slowly, notwithstanding the fact that it contains high concentrations of benzoyl-l-arginine amidase, leucine amino

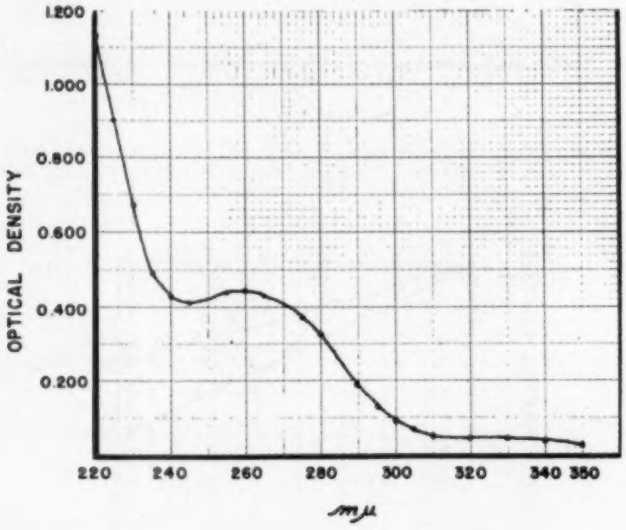


Chart 5.—Spectrophotometric analysis of a desoxyribonuclease inhibitor prepared from a cell-free extract of virulent tubercle bacilli (Ravenel strain).

peptidase and desoxyribonuclease. When, however, we exposed caseous material to an excess of enzymes in the form of cathepsin preparations from tuberculous rabbit spleen or liver, which also contained an abundance of desoxyribonuclease,[47] it was digested readily at pH 5.0.

Evidence for the Existence of a Desoxyribonuclease Inhibitor in Cell-Free Extracts of Tubercle Bacilli.—A bovine Ravenel strain of tubercle bacilli was cultivated in a liquid medium [51] for 30 days. The bacterial cells were precipitated in an International refrigerated centrifuge and washed three times with sterile, triple-distilled, pyrogen-free water (Abbott). During the third centrifugation, the high-speed attachment was used. The bacterial cells were transferred to a White

50. Weiss, C., and Singer, F. M.: Tr. Nat. Tuberc. A. **48**:1, 1952.

51. Crumb, C.: J. Bact. **52**:258, 1946.

bacteria-grinding mill after the addition of a few milliliters of distilled water. Steel balls, "globeads," and pulverized quartz sand were added; the grinding process was continued for three hours, and the cells were then spun down in the high-speed centrifuge. The cell-free supernatant fluid was kept in a frozen state at —45 C. for several days and then examined in a Beckman model DU spectrophotometer. Control material (distilled water) was similarly tested after being churned in the ball mill.

This cell-free extract of tubercle bacilli was found to exert strong inhibitory action on the desoxyribonuclease of normal and tuberculous lung tissue (Chart 4). Spectrophotometric analysis of the extract (Chart 5) revealed a peak at 260 mμ, suggesting the presence of breakdown products of desoxyribonucleic acid, while a sharp rise at 230 mμ and lower is indicative of spectra obtained with amino acids.[52] The curve resembles those of acid-soluble phosphorus compounds which are derived from autolysis of tissue homogenates and which contain purines and pyrimidines.

COMMENT

From a review of the literature of the past 20 years, it would appear that the processes involved in the mechanisms of caseation and softening of tubercles have not as yet been adequately defined from a chemical viewpoint. Referring first to caseation, various substances fractionated from tubercle bacilli—certain long chain fatty acids related to phthioic acid, as well as a lipopolysaccharide—have upon injections into animals brought about the development of necrosis, abscesses, and monocytic infiltration. It is not certain, however, whether these lesions should be regarded as reactions to a foreign body or as characteristic of progressive caseous tuberculosis. Moreover, only fragmentary data are available on the chemical anatomy of caseous material. It contains no soluble proteins but a very high percentage of cholesterol.[53] Certain proteolytic enzymes (benzoyl-l-arginine amidase, leucine amino peptidase) and desoxyribonuclease are present,[31] probably owing to the invasion of a varying number of phagocytic cells. Similarly, lipases, alkaline phosphatase,[30] and desoxyribonuclease remain in the caseous centers of tubercles, while acid phosphatase disappears. Notwithstanding this abundance of enzymes and substrates, caseous tubercles usually do not undergo autolysis in the manner characteristic of other types of necrotic tissue. When, for unknown reasons, autolysis and softening of tubercles have occurred, the contents of these tubercles are virtually free from proteinases, soluble proteins, desoxyribonuclease, and desoxyribonucleic acid. We see the end results of a vitally important phenomenon, but we are ignorant of its mechanism.

In the present communication, experimental data are reported which tend to show that there are enzyme inhibitors in the contents of caseous and softened tubercles. It is necessary, however, to isolate, identify, and characterize these inhibitors by modern immunochemical procedures. This work is now in progress in our laboratory. It has not yet been established whether these inhibitors have their origin in tubercle bacilli or whether the bacilli in the course of their destructive action cause the phagocytic and tissue cells to release their own enzyme inhibitors.[54] It

52. Saidel, L. J.; Goldfarb, A. R., and Waldman, S.: J. Biol. Chem. **197**:285, 1952.

53. Caldwell.[20] Cova.[21] Weiss and Schultz.[22]

54. Zamenhof, S., and Chargaff, E.: J. Biol. Chem. **180**:727, 1949. Henstell, H. H., and Freedman, R. I.: Science **115**:357, 1952. Henstell, H. H.; Freedman, R. I., and Ginsburg, B.: Cancer Res. **12**:346, 1952.

is important to clarify this situation, since, as Medlar [55] remarked, "In man it is evident that the phenomenon of necrosis with subsequent liquefaction and sloughing of the necrotic areas represents the major problem in the dissemination and in the complete eradication of the disease in the person affected."

SUMMARY AND CONCLUSIONS

The literature of the past 20 years dealing with the mechanism of caseation and softening of tubercles is reviewed.

A study is presented of the distribution of the enzyme desoxyribonuclease in various parts of discrete tubercles which were induced in the lungs of rabbits by means of the Wells apparatus for air-borne infection.

The inflammatory zones of tubercles and their caseous necrotic centers contain an abundance of desoxyribonuclease, probably originating from inflammatory cells. The contents of softened tubercles, on the other hand, show a striking reduction, or absence of desoxyribonuclease activity.

The desoxyribonuclease present in homogenates of rabbit tissues (lung, spleen, liver, and kidney) hydrolyzes desoxyribonucleic acid best at a pH of about 5.0, as compared with a pH optimum of about 7.0 for bovine pancreatic desoxyribonuclease.

In the contents of caseous and softened tubercles there are present substances which can inhibit the activity of certain proteinases (e. g., benzyl-l-arginine amidase) and of desoxyribonuclease. Investigation of the chemical and immunological nature of these substances is being continued.

Dr. Herbert L. Ratcliffe, Director of the Penrose Research Laboratory, Zoological Society of Philadelphia, infected the experimental animals.

55. Medlar, E. M.: Am. J. Med. **9**:611, 1950.

News and Comment

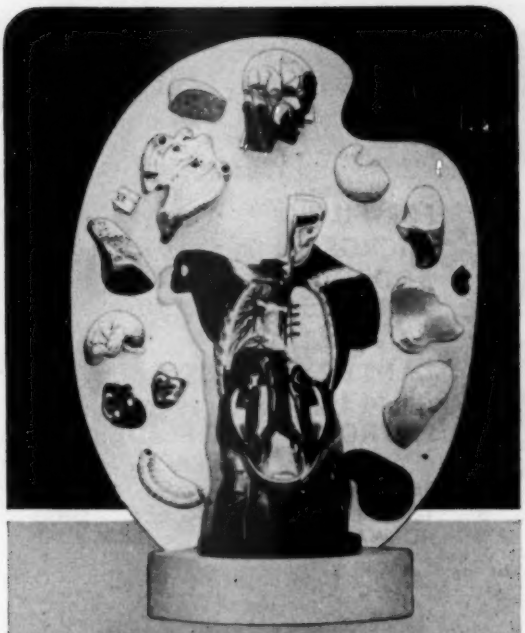
Ludvig Hektoen Memorial Lecture.—The first Ludvig Hektoen Memorial Lecture was presented on May 14, 1953, by Paul Klemperer, M.D., of New York City. The title of Dr. Klemperer's address was "Collagen Disease—Evolution of the Concept." This lecture was sponsored by the Hektoen Institute for Medical Research of the Cook County Hospital, Chicago.

Sociedad Venezolana de Anatomia Patologica.—Notice has been received of the establishment of the Sociedad Venezolana de Anatomia Patologica, in Caracas, Venezuela. Officers of the society for the current year are President, J. A. O'Daly; Treasurer, L. Potenza, and Secretary, Alberto Rivero.

New Appointment for Dr. McManus.—The appointment of J. F. A. McManus, M.D., as Professor and Chairman of the Department of Pathology at the University of Alabama College of Medicine has been announced. Since 1950, Dr. McManus has served as Associate Professor of Pathology at the University of Virginia.

CORRECTION

In the running head for the article entitled "Classification of Glandular Tumors of Salivary Glands" by Drs. William H. Bauer and John D. Bauer, published in the April issue, page 328, the word "Granular" was incorrectly substituted for the word "Glandular."



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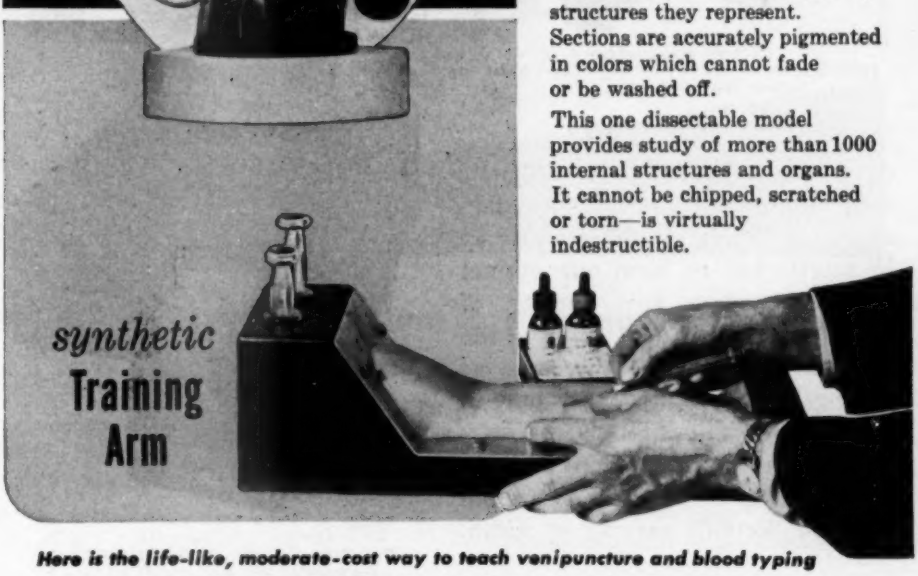

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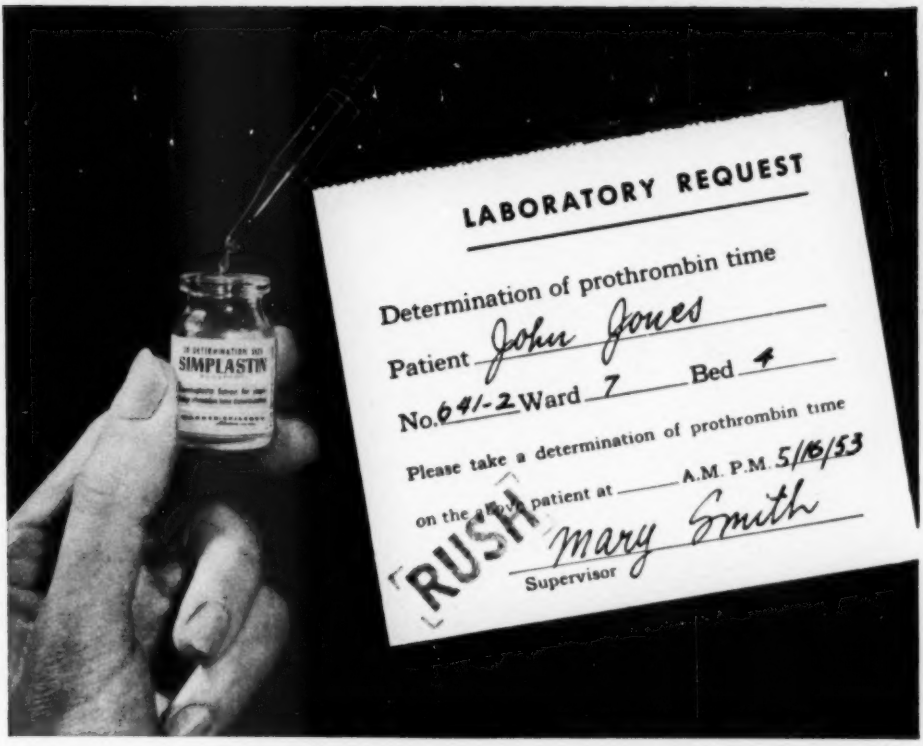

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